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**WO 02/068610 A2**

(54) Title: COMPOSITIONS AND METHODS FOR AFFECTING VIRULENCE DETERMINANTS IN BACTERIA

(57) Abstract: A novel *sar R* gene and *sar R* gene product which down regulates the expression of *sar A* and the resulting virulence determination in *Staphylococcus aureus* is provided. Methods for modulating the expression of *sar A* and virulence determinants are also provided. A preferred embodiment of the present invention provides structural information relating to the gene product and enables the identification and formulation of lead compounds and reducements for treating and preventing infections by *S. aureus* and related bacteria.

**Compositions and Methods for Affecting Virulence Determinants in Bacteria**

This application claims priority to U.S. Provisional Application Serial No. 60/261,233, filed January 12, 2001, U.S. Provisional Application Serial No. 60/261,607, filed January 12, 2001, and U.S. Provisional Application Serial No. 5 60/289,601, filed May 8, 2001. These applications are hereby incorporated by references herein in their entirety.

**Field of the Invention**

The present invention relates generally to the field of molecular biology. More particularly, certain embodiments concern methods and compositions 10 comprising DNA segments and protein derived from *Staphylococcus aureus* and other bacterial species. The present invention also relates to the three-dimensional structure of proteins derived from *S. aureus* and other bacterial species and methods of identifying and developing pharmaceuticals using, among other things, drug screening assays.

**15 Background of the Invention**

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications is found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this 20 application.

Bacterial infections are a serious problem in humans. In the past decade, the number of "supergerms" that resist treatment has increased dramatically. Unfortunately, the very same arsenal of drugs used to overcome these microbes helped give rise to antibiotic-resistant strains of bacteria. Of great importance are 25 several antibiotic-resistant and sometimes fatal bacteria including *S. aureus*, *Pseudomonas aeruginosa* (pneumonia), and *Enterococcus faecalis* (urinary tract and blood infections).

For most healthy people, these antibiotic-resistant bacteria are not life-threatening. The immune system, the body's natural defense against microbes, 30 usually fights off disease-causing bacteria. However, when bacteria attack people with weakened immune systems they can be deadly. Hailed as miracle drugs,

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antibiotics have cured thousands of bacterial infections, from acne to strep throat to ear infections. Today, there are more than 100 types of antibiotics on the U.S. market. Due to increasing resistance to antibiotics, however, new treatments are still needed.

In particular, *S. aureus* infections have been problematic to treat.

- 5     *S. aureus* are non-mobile, non-sporulating gram-positive cocci 0.5-1.5 $\mu$ m in diameter, that occur singly and in pairs, short chains, and irregular three-dimensional grape-like clusters. *S. aureus* can grow over a wide range of environmental conditions, but they grow best at temperatures between 30°C and 37°C and at a neutral pH. They are resistant to desiccation and to chemical disinfection, and they tolerate NaCl
- 10    concentrations up to 12%. It has been found that the growth of *S. aureus* becomes unusually sensitive to high NaCl concentrations (by decreasing Ca<sup>2+</sup> concentration) in growth media allowing for autolysis (29).

*S. aureus* can cause a wide spectrum of infections ranging from superficial abscesses, pneumonia and endocarditis to sepsis (4). The ability of *S. aureus* to cause a multitude of human infections is due, in part, to an impressive array of extracellular and cell-wall associated virulence determinants that are coordinately expressed in this organism (51). The coordinate expression of many of these virulence determinants in *S. aureus* and other bacteria is regulated by global regulatory elements such as *sarA* and *agr* (15, 34). These regulatory elements, in turn, control the transcription of a wide variety of unlinked genes many of which have been implicated in pathogenesis.

      The global regulatory locus *agr* encodes a two-component, quorum sensing system that is involved in the generation of two divergent transcripts, RNAII and RNAIII, from two distinct promoters, P2 and P3, respectively. RNAIII is the regulatory molecule of the *agr* response, hence responsible for the up-regulation of extracellular protein production and down-regulation of cell-wall associated protein synthesis during the postexponential phase (39,49). The RNAII molecule, driven by the P2 promoter, encodes a four-gene operon, *agrBDCA*, with AgrC and AgrA corresponding to the sensor and activator proteins of a two component regulatory system. Additionally, *agrD*, in concert with *agrB*, participates in the generation of an octapeptide with quorum sensing functions (31,41). The autoinducing peptide would

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stimulate the transcription of the *agr* regulatory molecule RNAIII which ultimately interacts with target genes to modulate transcription (49) and possibly translation (44).

In contrast to *agr*, the *sarA* locus activates the synthesis of both extracellular (e.g.  $\alpha$ - and  $\beta$ - hemolysins) and cell-wall proteins (e.g. fibronectin binding protein) in *S. aureus* (15). The *sarA* locus is composed of three overlapping transcripts [*sarA* P1 (0.56 kb), *sarA* P3 (0.8 kb) and *sarA* P2 (1.2 kb) transcripts], each with a common 3' end but initiated from three distinct promoters (P1, P3 and P2 promoters). Due to their overlapping nature, each of these transcripts encodes the major 372-bp *sarA* gene, yielding the 14.5 kDa *sarA* protein (2). DNA footprinting studies have shown that the *sarA* protein binds to the promoters of several target genes (19) including *agr*, *hla* (alpha hemolysin gene), *spa* (protein A gene) and *fnbA* (fibronectin binding protein A gene), thus implicating *sarA* as a regulatory molecule that can modulate target gene transcription via both *agr*-dependent and *agr*-independent pathways (9,19,20). With *agr*-dependent pathway of target gene activation, the *sarA* protein binds to the *agr* promoter to stimulate RNAIII transcription and RNAIII, in turn, interacts with target genes (e.g. *hla*) to modulate transcription. With *sarA*-dependent but *agr*-independent pathway, the SarA protein will interact directly with target-gene promoters (e.g. *hla* and *spa*) (19) to control gene transcription. Deletion and promoter fusion analyses indicates that the regions upstream of the *sarA* P2 and between the P1 and P3 promoters have a modulating role in *sarA* expression, possibly by controlling transcription from the *sarA* P1 promoter, the predominant promoter within the *sarA* locus (10,39) (Fig. 1A).

A great need exists for methods and compositions which can affect or regulate the virulence of bacteria, such as the expression of *sarA* and the resultant virulence determinants of *S. aureus* and other bacteria.

#### Summary of the Invention

The present invention provides a new genetic locus of *S. aureus* and other bacteria. The gene at this locus is referred to herein as *sarR*. The *sarR* gene is involved in the regulation and expression of virulence determinants in *S. aureus* and other bacteria.

The present invention provides a polynucleotide sequence of the *sarR* gene (SEQ. ID. NO: 1) which is involved in the regulation and expression of

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virulence determinants in *S. aureus* and related bacteria. Also provided is a *sarR* gene product, an isolated polypeptide which is involved in regulation and expression of virulence determinants in *S. aureus* and related bacteria.

An object of the present invention is to provide nucleic acid sequences  
5 that regulate the expression of virulence determinants in *S. aureus* and related bacteria. In a preferred embodiment the nucleic acid sequence is isolated from *S. aureus* and in another preferred embodiment the nucleic acid sequence is a *sarR* gene (SEQ. ID. NO: 1).

Another object of the present invention is to provide a polypeptide  
10 which regulates the expression of virulence determinants in *S. aureus* and other bacteria. In a preferred embodiment the polypeptide is isolated from *S. aureus* and in another preferred embodiment the polypeptide is encoded by a *sarR* gene and correspond to the sequence set forth in SEQ ID NO: 2.

Vectors and host cells comprising nucleic acid sequences encoding  
15 these polypeptides and chemical entities that mimic or enhance the activity of such polypeptides are further objects of the invention. These agents can be used alone or in combination with antibacterial agents such as penicillin to enhance the properties of such agents.

Another object of the present invention is to provide methods for  
20 modulating regulation and expression of virulence determinants in *S. aureus* and related bacteria to inhibit their growth and infectivity by contacting the bacteria with an agent which interacts with *sarA* promoter regions.

Another object of the present invention is to provide a method of  
inhibiting growth and infectivity of bacteria comprising contacting the bacteria with  
25 an agent which enhances the expression of the *sarR* gene, or the activity of a polypeptide encoded by the *sarR* gene.

Another object of the present invention is to provide a kit for identifying the presence of a *sarR* gene or a polypeptide encoded by a *sarR* gene.

A further object of the present invention is to provide nucleic acid  
30 sequences from a mutant *sarR* gene in *S. aureus* and related bacteria, and polypeptides encoded by a mutant *sarR* gene.

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A further object of the invention is to provide a three dimensional crystal structure of the SarR protein.

A further object of the invention is to provide methods of screening for lead compounds which inhibit the expression of virulence determinants in *S. aureus* and related bacteria.

Another object of the present invention is to provide pharmaceutical compositions for use as anti-bacterial agents against *S. aureus* and other bacteria.

#### Brief Description of the Drawings

Figure 1A illustrates a schematic of the *sar* promoters and transcripts.

Figure 1B shows the promoter region of *sarR*. The transcription start site has been mapped by primer extension to position 119. Examples of -10 and -35 promoter boxes are in bold and underlined.

Figure 1C shows the alignment of SarR with SarA. Colon represents identity while a period indicates conservative substitution.

Figure 2 demonstrates purification of *sarR* from the pET11b expression vector.

Figure 3A demonstrates PCR amplification of *sarR*-like genes in *S. aureus* strains RN6390, Cowan I, DB and Newman, *S. epidermidis*, *S. haemolyticus* and *S. saprophyticus*.

Figure 3B shows a Southern blot of the strains in Fig. 3A above strain restricted with *Cla*I and probed with a 345-bp *sarR* probe (nt 208-552).

Figure 3C shows a Northern blot of the total cellular RNA (10 µg each) of the strains of Fig. 3D probed with a *sarR* probe.

Figure 3D shows cell lysates of the above strains immunoblotted onto nitrocellulose and probed with anti-*sarR* monoclonal antibody 2A7 at a 1:2000 dilution.

Figure 4 shows gel shift assays of end-labeled  $^{32}\text{P}$  fragment of *sarA* P1 (nt 531-859) (2), P2 (nt 1-196) and P3 (nt 364-525) promoters.

Figure 5 shows DNaseI footprinting assays of *sarR* with end-labeled  $^{32}\text{P}$  *sar* P2 (49-bp fragment), P1 (nt 531-859) and P1' (nt 620-859) promoter fragments.

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Figure 6 shows the expression of *sarR* in parental strain RN6390 and its *sar* (ALC488) and *agr* (RN6911) mutants. Figure 6A illustrates Northern blots of the *sarR* transcript in RN6390 and its isogenic *sar* and *agr* mutants. Figure 6B illustrates ethidium bromide stain of an RNA gel prior to transfer to hybridization membrane. Figure 6C illustrates the expression of *sarR* on an immunoblot probed with anti-*sarR* antibody 2C7.

Figures 7A & B illustrate promoter activation of *sarA* P1 and combined P2-P3-P1 promoters fused to a *gfp<sub>uvr</sub>* reporter gene as evaluated in a fluorescence spectrophotometer (FL600, BioTek Instrument).

Figure 8 demonstrates the effect of the *sarR* mutation on *sarA* and *agr* expression. Figure 8A illustrates *sarA* expression during mid-log and late-log phases and early stationary phases. Figure 8B illustrates *agrA* (RNAII) transcription. It is a Northern blot of the RNAII (*agrA* probe) transcript in RN6390 and the *sarR* mutant (10 µg of total RNA each). The *agrA* probe corresponds to nt 3830-4342 according to published sequence (23).

Figure 9A illustrates a ribbon (8) diagram of the three-dimensional structure of the SarR-MBP fusion protein. The SarR dimer is at the top colored Green and Yellow for each monomer, respectively, two MBP molecules are at bottom colored Blue and Pink, respectively.

Figure 9B illustrates a ribbon diagram of the three-dimensional structure of the SarR monomer. Starting from the N-terminus,  $\alpha_1$  (6-24) →  $\alpha_2$  (32-44) →  $\beta_1$  (47-50) →  $\alpha_3$  (residue 51-58) →  $\alpha_4$  (63-74) →  $\beta_2$  (79-83) →  $\beta_3$  (90-96) →  $\alpha_5$  (97-113).  $\alpha_2$ ,  $\beta_1$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\beta_2$ , and  $\beta_3$  are defined as a "winged helix motif".  $\alpha_3$  →  $\alpha_4$  represent a helix-turn - helix-motif.

Figure 10A illustrates one view of the structure of the SarR dimer. One SarR monomer is colored Green while the other is Yellow. This top view of the concave side along the dimer 2-fold axis of the SarR dimer, subdomain1 contains  $\beta_1$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\beta_2$ ,  $\beta_3$  (labeled blue) from one monomer, subdomain 2 contains  $\beta_1$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\beta_2$ ,  $\beta_3$  (also labeled blue) from the other, subdomain 3 contains  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_5$  (labeled red) from both monomers.

Figure 10B illustrates a middle view perpendicular to the dimer 2-fold.

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Figure 10C illustrates a bottom view of the convex side of the SarR dimer. N1 and C1 are the N terminus and C terminus of molecule 1. N2 and C2 are termini of molecule two.

Figure 11 illustrates a sequence alignment of Sar homologs from *S. aureus*. The sequences are presented in one-letter amino acid code. Numbers at the beginning of each line indicate amino acid positions relative to the start of each protein sequence. Helices are indicated by rectangles,  $\beta$ -sheets are indicated by arrows, and loops are indicated by a line. "H" marked with Green represents residues that take part in dimerization. "A" marked with Red represents residues that may compose of the activation motifs. "D" marked with Blue represents residues that is involved in the interactions of *sarR* with DNA. The sequence of DNA binding motif from CAP is also aligned to the Sar proteins. Dot means residue or residues omitted, dash means residue or residues missed in CAP. SarS2 starts at 1 (actual position on SarS is 126).

Figures 12A and 12B illustrate the electrostatic potential surface of the *sarR* dimer calculated by GRASP (46), with charge +1 for Lys and Arg, charge -1 for Glu and Asp, and charge zero for all other residues. The color bar from red to blue represents potential from negative to positive defined as in GRASP. The Blue represents positive charged potential, Red represents negative charged potential.

Figure 12A illustrates the potential surface of the concave side of SarR dimer (similar orientation as Fig. 10A). The direct line distance of AB is ~65 $\text{\AA}$ ; the distance of the inner surface covered by AB is 90 $\text{\AA}$ . Figure 12B illustrates the potential surface of the convex side of the SarR dimer (similar orientation as Fig. 10C). Two aspartic acid residues are from one molecule, three glutamic acid residues from the other.

Figure 13A illustrates the superposition of two "winged helix motifs" (subdomain 1 plus  $\alpha$ 2 from one monomer and subdomain 2 plus  $\alpha$ 2 from another monomer) of SarR dimer with the DNA binding domains of catabolite activating protein (CAP, PDB ID 1cgp); subdomains from SarR are marked Green and Yellow respectively; subdomains from CAP are marked Blue and Red;  $\alpha$ 4 and W1 interact with DNA at major grooves and minor grooves respectively.

Figure 13B illustrates the DNA binding model of SarR and DNA (similar orientation as Fig. 10A and Fig. 13A). The SarR dimer is superimposed to

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that of CAP. The DNA structure is from the CAP-DNA complex structure (PDB ID 1cgp). The helix-turn - helix-motif binds to the major groove while the wing region (W1) conformation is slightly adjusted to fit in the minor groove;

Figure 13C illustrates a 90° orientation from view of Fig. 13B.

## 5    Detailed Description of the Invention

If appearing herein, the following terms shall have the definitions set out below.

As used herein, the term "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids.

10           A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment is attached so as to bring about the replication of the attached segment.

15           A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus

20           A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site as well as protein 25 binding domains (consensus sequences) responsible for the binding of RNA polymerase.

25           As used herein, the term "homologous" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies and homologous proteins from different species. Such proteins have sequence homology as reflected by their high degree of sequence similarity. Preferably, homologous gene sequences will have at least 50% sequence identity (as defined by the maximal base match in a computer-

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generated alignment of two nucleic acid sequences), more preferably at least 60%, and most preferably at least 80%. For polypeptide gene products of such homologous genes, generally the gene products also exhibit a significant degree of amino acid sequence identity. Thus, for such polypeptide products of homologous genes, the 5 amino acid sequences have at least 25% sequence identity over a sequence of 100 or more amino acids, more preferably at least 40%, still more preferably at least 60%, and most preferably at least 80%. In addition, in the present context, the products of the homologous gene sequences are also involved in regulation of *sarA* expression. Thus, the present invention applies as well to bacteria having significant sequence, 10 structural, or functional homology to the *sarR* gene or SarR protein.

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin. However, in common usage and in the instant application, the term 15 "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. Thus, the term 20 "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

The term "a sequence essentially as set forth in SEQ ID NO: --" means that the sequence substantially corresponds to a portion of SEQ ID NO:-- and has relatively few amino acids that are not identical to, or a biologically functional 25 equivalent of, the amino acids of SEQ ID NO:--. Accordingly, sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:-- will be sequences that are "essentially as set forth in SEQ ID NO:--."

30 Typically, though not necessarily, a ligand in the context of this invention is a molecule with a molecular weight of less than 10,000 daltons, more typically less than 5,000 daltons.

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As used herein "related bacterial species" refer to bacterial species having a gene corresponding to the sequence as essentially set forth in SEQ ID NO: 1, and analogs or homologs thereof. Related bacteria according to the present invention include *S. aureus* strains RN6390, Newman, Cowan I and DB and *S. saprophyticus*.

5 The present invention provides a novel gene found in *S. aureus* and other bacterial species. This gene is designated herein as *sarR* and is represented SEQ ID NO: 1. The present invention also provides for a novel gene corresponding to the sequence as essentially set forth in SEQ ID NO: 1, and analogs or homologs thereof.

The present invention also provides the *sarR* gene product designated  
10 herein as the SarR protein represented in SEQ ID. NO. 2, and analogs or homologs thereof. The SarR protein has a molecular weight of approximately 13.7 kDa and has a deduced basic pI of approximately 9.2. The *sarR* gene product is also characterized by a predominance of charged residues (34%).

The present invention also relates to newly discovered binding sites in  
15 the *sarA* promoter system. These binding sites enable the SarR protein to repress *sarA* expression by preventing *sarA* from initiating the expression of virulence factors

The present invention further provides a model for a SarA/SarR heterodimer which interferes with the function of the SarA homodimer. The present invention also provides alternative heterodimers which include SarA/SarR  
20 peptidomimetic combinations and other combinations resulting from lead compounds developed from structural analysis of SarR.

The present invention also provides a *sarR* gene product or peptidomimetic capable of directly binding to promoters of a virulence gene such as the *hla* promoter of the alpha hemolysin gene to provide direct inhibition of such  
25 virulence factors.

The present invention also provides a method of down regulating *sarA* P1 transcription to repress the expression of *sarA*. This function of the *sarR* gene product was demonstrated by the results of mutant studies disclosed herein. The *sarR* mutant expressed a much higher level of the SarA protein than the parent strain.  
30 Accordingly, the present invention also provides *sarR* mutant strains. These strains are useful as experimental and diagnostic tools to specifically characterize the *sarA* expression systems:

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The present invention also provides a detailed three-dimensional (3-D) crystal structure of the SarR protein. The structural data demonstrate that the protein is a member of a new family of winged helix proteins.

The present invention provides methods for treating microbial pathogenesis by the administration of SarR analogs in the form of small molecule compounds which alter the functioning of a microbial *sarA* expression. Reducing or eliminating the expression of *sarA* by such analogs can greatly alter the course and effects of a bacterial infection. This treatment approach is distinct from most prior bactericidal or bacteriostatic antimicrobial treatments which attempted to either kill the microbial cells, or directly prevent them from reproducing. The antimicrobial action of the compounds of these standard antimicrobial treatments is exerted both *in vivo*, in an infection, and *in vitro*, in a culture, unless some specific compensating factor(s) is provided which allows survival or growth in the presence of the antimicrobial agent. In contrast, this invention is directed at the regulation of *sarA* expression which is involved in the pathogenesis process, but is not necessarily essential for microbial survival or growth *in vitro*. By selectively targeting the expression of virulence factors and not directly killing the bacterium there is less pressure to develop antimicrobial resistance. Because this type of therapy is less likely to alter the local flora, the incidence of superinfection (e.g. fungal or other bacterial infections such as *pseudomonas* or *enterococcus*) is reduced.

According to one aspect of the present invention, an analog library is provided to produce a very large number of potential molecules for regulating the *sarA* expression system, and in general the greater the number of analogs in the library, the greater the likelihood that at least one member of the library will effectively regulate the *sarA* expression system. Designed libraries following a particular template structure and limiting amino acid variation at particular positions are much preferred, since a single library can encompass all the designed analogs and

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the included sequences will be known and presented in roughly equal numbers. By contrast, random substitution at only six positions in an amino acid sequence provides over 60 million analogs, which is a library size that begins to present practical limitations even when utilizing screening techniques as powerful as phage display.

- 5    Libraries larger than this would pose problems in handling, e.g., fermentation vessels would need to be of extraordinary size, and more importantly, the likelihood of having all of the planned polypeptide sequence variations represented in the prepared library would decrease sharply. It is therefore preferred to create a designed or biased library, in which the amino acid positions designated for variation are considered so
- 10   as to maximize the effect of substitution on the *sarA* regulation characteristics of the analog, and the amino acid residues allowed or planned for use in substitutions are limited.

The use of replicable genetic packages, such as the bacteriophages, is one method of generating novel polypeptide entities that regulate *sarA* expression.

- 15   This method generally consists of introducing a novel, exogenous DNA segments into the genome of a bacteriophage (or other amplifiable genetic package) so that the polypeptide encoded by the non-native DNA appears on the surface of the phage. When the inserted DNA contains sequence diversity, then each recipient phage displays one variant of the template (parental) amino acid sequence encoded by the
- 20   DNA, and the phage population (library) displays a vast number of different but related amino acid sequences.

Such techniques make it possible not only to screen a large number of potential binding molecules but make it practical to repeat the binding/elution cycles and to build secondary, biased libraries for screening analog-displaying packages that

25   meet initial criteria.

It is well-known to those normally skilled in the art that it is possible to replace peptides with peptidomimetics. Peptidomimetics are generally preferable as therapeutic agents to peptides owing to their enhanced bioavailability and relative lack of attack from proteolytic enzymes. Techniques of molecular modeling may be

30   used to design a peptidomimetics which mimic the structure of the SarR peptide disclosed herein. Accordingly, the present invention also provides peptidomimetics and other lead compounds which can be identified based on the data obtained from

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structural analysis of the SarR protein disclosed herein. A potential SarR analog is examined through the use of computer modeling using a docking program such as GRAM, DOCK, or AUTODOCK. This procedure can include computer fitting of potential SarR analogs. Computer programs can also be employed to estimate the 5 attraction, repulsion, and steric hindrance of an analog to a potential binding site. Generally the tighter the fit (e.g., the lower the steric hindrance, and/or the greater the attractive force) the more potent the potential drug will be since these properties are consistent with a tighter binding constant. Furthermore, the more specificity in the 10 design of a potential drug the more likely that the drug will not interfere with other properties of the *sarA* expression system. This will minimize potential side-effects due to unwanted interactions with other proteins.

Initially a potential SarR analog could be obtained by screening a random peptide library produced by a recombinant bacteriophage, for example, or a chemical library. A analog ligand selected in this manner could be then be 15 systematically modified by computer modeling programs until one or more promising potential ligands are identified.

Such computer modeling allows the selection of a finite number of rational chemical modifications, as opposed to the countless number of essentially random chemical modifications that could be made, and of which any one might lead 20 to a useful drug. Thus through the use of the three-dimensional structure disclosed herein and computer modeling, a large number of compounds is rapidly screened and a few likely candidates can be determined without the laborious synthesis of untold numbers of compounds.

Once a potential SarR analog is identified it can be either selected from 25 a library of chemicals commercially available from most large chemical companies including Merck, GlaxoWelcome, Bristol Meyers Squib, Monsanto/Searle, Eli Lilly, Novartis and Pharmacia UpJohn, or alternatively the potential ligand is synthesized *de novo*. As mentioned above, the *de novo* synthesis of one or even a relatively small group of specific compounds is reasonable in the art of drug design.

30 In a further aspect, this invention provides methods of treating a bacterial infection by administering a pharmaceutical composition comprising a SarR analog, peptide, or peptidomimetic to inhibit the expression of *sarA*. The present

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invention also provides a method for prophylactic treatment of a mammal, in particular a human, in order to prevent a bacterial infection. Such treatment comprises administering a pharmaceutical preparation comprising a SarR analog, peptide, or peptidomimetic to the mammal. Preferably such treatment would be used  
5 when the patient is at risk of contracting or developing a bacterial infection. Such a prophylactic treatment method may have particular benefit, for example, for treating patients prior to surgical operations.

Pharmaceutical compositions according to the present invention may comprise peptides and peptidomimetics of the present invention in association with a  
10 pharmaceutically acceptable carrier or excipient, adapted for use in human or veterinary medicine. The compositions may contain from 0.001-99% of the active material. Such compositions may be presented for use in conventional manner in admixture with one or more physiologically acceptable carriers of excipients. The pharmaceutical compositions according to the invention may also contain other active  
15 ingredients such as antimicrobial agents, or preservatives. The compositions may optionally further contain one or more other therapeutic agents which may, if desired, be a chemotherapeutic antiviral agent.

Pharmaceutically acceptable salts of the peptides of this invention may be formed conventionally by reaction with an appropriate acid. The additional salts so  
20 formed from addition by acid may be identified by hydrochloric, hydrobromic, phosphoric, acetic, fumaric, salicylic, citric, lactic, mandelic, tartaric, oxalic, methanesulfonic, and the like.

Thus, the peptides and peptidomimetics according to the present invention may be formulated for oral, buccal, parenteral, topical or rectal  
25 administration. In particular, these peptides and peptidomimetics may be formulated for injection or for infusion and may be presented in unit dose form in ampoules or in multidose containers with an added preservative. The compositions may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.  
30 Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g. sterile, pyrogen-free water, before use.

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The present invention further provides a process for preparing a pharmaceutical composition which comprises bringing a peptide or peptidomimetic of the invention into association with a pharmaceutically acceptable excipient or carrier.

The dosage of the peptide or peptidomimetic used in the treatment will  
5 vary, depending on the seriousness of the disorder, the weight of the patient, the relative efficacy of the peptide and the judgment of the treating physician. Unit dosages may be administered more than once a day, e.g., two or three times a day. Such therapy may extend for several weeks, in an intermittent or uninterrupted manner, until the patient's symptoms are eliminated.

10 The present invention also provides pharmaceutical compositions which comprise a pharmaceutically effective amount of the peptides of this invention, or pharmaceutically acceptable salts thereof, and, preferably, a pharmaceutically acceptable carrier or adjuvant. Therapeutic methods of this invention comprise the step of treating patients in a pharmaceutically acceptable manner with those peptides  
15 or compositions. Such compositions may be in the form of tablets, capsules, caplets, powders, granules, lozenges, suppositories, reconstitutable powders, or liquid preparations, such as oral or sterile parenteral solutions or suspensions.

In order to obtain consistency of administration, it is preferred that a composition of the invention is in the form of a unit dose. The unit dose presentation  
20 forms for oral administration may be tablets and capsules and may contain conventional expedients. For example binding agents, such as acacia, gelatin, sorbitol, or polyvinylpyrrolidone; fillers, such as lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tabletting lubricants such as magnesium stearate; disintegrants, such as starch, polyvinylpyrrolidone, sodium starch glycolate or  
25 microcrystalline cellulose; or pharmaceutically acceptable wetting agents such as sodium lauryl sulfate.

The solid oral compositions may be prepared by conventional methods of blending, filling, tabletting, or the like. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large  
30 quantities of fillers. Such operations are, of course, conventional in the art. The tablets may be coated according to methods well-known in normal pharmaceutical practice, in particular with an enteric coating.

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Oral liquid preparations may be in the form of emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may or may not contain conventional additives. For example suspending agents, such as sorbitol, syrup,

5      methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethylcellulose, aluminum stearate gel, or hydrogenated edible fats; emulsifying agents, such as sorbitan monooleate or acacia; non-aqueous vehicles (which may include edible oils), such as almond oil, fractionated coconut oil, oily esters selected from the group consisting of glycerine, propylene glycol, ethylene glycol, and ethyl alcohol; preservatives, for

10     instance methyl para-hydroxybenzoate, ethyl para-hydroxybenzoate, n-propyl parahydroxybenzoate, or n-butyl parahydroxybenzoate or sorbic acid; and, if desired, conventional flavoring or coloring agents.

For parenteral administration, fluid unit dosage forms may be prepared by utilizing the peptide and a sterile vehicle, and, depending on the concentration

15     employed, may be either suspended or dissolved in the vehicle. In preparing solutions, the peptides of this invention may be dissolved in water, whereas opiates used heretofore showed only marginal solubility in aqueous media or physiological fluids. Once in solution, the peptide may be injected and filter sterilized before filling a suitable vial or ampoule and subsequently sealing the carrier or storage package.

20     Adjuvants, such as a local anaesthetic, a preservative or a buffering agent, may be dissolved in the vehicle prior to use. Stability of the pharmaceutical composition may be enhanced by freezing the composition after filling the vial and removing the water under vacuum, e.g., freeze drying the composition. Parenteral suspensions may be prepared in substantially the same manner, except that the peptide should be

25     suspended in the vehicle rather than being dissolved. A surfactant or wetting solution may be advantageously included in the composition to facilitate uniform distribution of the peptide.

#### **Experimental Materials, Methods and Results**

**Bacterial Strains and Growth Conditions.** As way of example,

30     certain bacterial strains and plasmids used herein are listed in Table 1 as shown below.

**Table 1**

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Strains or plasmid	References	Comments
S. aureus		
RN4220	(32)	A mutant of 8325-4 that accepts DNA
RN6390	(32)	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocyte agar (parental strain)
RN6911	(34)	An agr mutant of RN6390 with an agr::tetM mutation
ALC488	(9)	A sarA mutant with a sarA::ermC mutation
ALC1713	this study	A sarR mutant of RN6390 with a sarR::ermC mutation
Cowan I	(17)	A laboratory strain
DB	(10)	A clinical blood isolate previously used in adhesion and endocarditis studies
Newman	(29)	A laboratory strain
S. epidermidis		A strain from the collection at the Utrecht University Hospital
S. haemolyticus		A strain from the collection at the Utrecht University Hospital
S. saprophyticus		A strain from the collection at the Utrecht University Hospital
E. coli		
XL-1 blue	(26)	A host strain for cloning
DH5 $\alpha$	(26)	A host strain for cloning
Plasmids		
pCR2.1	Invitrogen	E. coli cloning vector for direct cloning of PCR products
pBluescript	Stratagene	E. coli cloning vector
pUC18	(26)	E. coli cloning vector
pACYC177	New England BioLabs	E. coli cloning vector
pCL52.1	(24)	A temperature sensitive E. coli/S. aureus shuttle vector
pET11b	Novagen	Expression vector for E. coli
pALC926	this study	pUC18 containing a 49-bp fragment

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Strains or plasmid	References	Comments
		upstream of the P2 promoter of the <i>sarA</i> locus
pALC1357	this study	pET11b containing the 345-bp <i>sarR</i> gene at the NdeI/BamHI site
pALC1361	this study	pACYC177 with ~4 kb <i>Clal</i> fragment containing the <i>sarR</i> region of RN6390
pALC1627	this study	pBluescript with a 2.5 kb <i>EcoRI/Clal</i> fragment containing the <i>sarR</i> gene subcloned from pALC1361
pALC1687	this study	pBluescript with a 290-bp deletion of the <i>sarR</i> gene in pALC1627
pALC1696	this study	pCL52.1 with a 290-bp <i>sarR</i> deletion replaced by the <i>ermC</i> gene at the <i>EcoRV/SalI</i> site

Phage Φ11 was used as the transducing phage for *S. aureus* strains. *S. aureus* strain RN4220, a restriction-deficient derivative of strain 8325-4 (47), was used as the initial recipient for the transformation of plasmid construct by 5 electroporation, following the protocol of Schenk and Laddaga (58).

*S. aureus* cells were grown at 37°C with aeration in CYGP or 03GL broth (47,48) or tryptic soy broth supplemented with antibiotics when necessary. 03GL and NYE agar (58) containing antibiotics were routinely used for the selection of *S. aureus* transformants. Luria- Bertani medium was used for growing *Escherichia coli*. Antibiotics were used at the following concentrations: erythromycin, 5 µg/ml; tetracycline, 5 µg/ml and chloramphenicol, 10 µg/ml for *S. aureus*; and ampicillin, 50 µg/ml; chloramphenicol, 30 µg/ml; erythromycin, 200 µg/ml and spectinomycin, 75 µg/ml for *E. coli*.

**Cloning of the *sarR* gene and construction of the *sarR* mutant.** The 15 SarR protein was partially purified from crude cell lysate by passing the lysate over a DNA-specific column containing a 49-bp DNA fragment (nt 71-119) covalently linked to Sepharose (18). The first 14 residues of the SarR protein in the amino-terminus were determined by microsequencing at the Core Facility at our institution. A blast search of the *S. aureus* genome data bank at TIGR revealed a

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partial ORF of 47 amino acids. Using these data, a 141-bp fragment with two degenerate oligos was amplified by PCR.

(5'-ATG T/A C/G A/T AAAAT T/C AA T/C GATAT T/C AA T/C GATTTC-3')

(SEQ. ID NO: 4);

5 (5'-ATT T/A G/C A/T T/C TC T/A G/C A/T A/T C G/T T/C AA A/G AT A/G TG  
A/G TT T/C AA -3') (SEQ. ID NO. 5) The PCR fragment was cloned into the vector  
pCR2.1 (Invitrogen). Southern hybridization of enzyme-restricted chromosomal  
DNA of the parental strain RN6390 with a radiolabeled 141-bp DNA probe revealed a  
single ~ 4 kb *Cla* I-digested hybridizing fragment. To clone this fragment, *Cla*I-  
10 digested chromosomal DNA in the range of 3-5 kb was resolved in an agarose gel,  
excised, purified and ligated to the *Cla*I site of pACYC177 in *E. coli* DH5 $\alpha$ .  
Positive-reacting clones were identified, all containing the ~4 kb *Cla*I fragment. One  
of these clones was sequenced, revealing a 345-bp ORF with identity to the partial 47  
amino acid sequence of *sarR* as predicted from the *S. aureus* genome.

15 Deletion and insertion mutagenesis was performed with the Stratagene Quick Change kit to introduce a deletion and a mutation concomitantly into the *sarR* gene. In brief, the ~4 kb *Cla*I DNA fragment containing the *sarR* gene in recombinant pACYC177 was cloned into pBluescript to serve as a template for mutagenesis. The following oligonucleotide

20 (5'-22GCATAAAAAGATATCGGGCATT45-338GTGAGTCTAACGATATCTCATCTAAA<sup>363</sup>  
-3')

T T A

[SEQ ID. NOS: 6 and 7]

and its complement were used to construct a deletion and to introduce an exogenous  
25 *EcoRV* restriction site into the *sarR* gene (restriction site underlined, with the native  
nucleotides displayed below, intact *sarR* gene from nt 208-555). After amplification  
with the recombinant pBluescript template, the PCR product was digested with *Dpn*I  
to remove methylated template DNA (i.e. pBluescript with the native *sarR* gene) and  
transformed into XL1-Blue cells to select for ampicillin-resistant colonies. Successful  
30 deletion and mutation in the resultant clones were confirmed by restriction analysis  
with *EcoRV* and finally verified by automated DNA sequencing. The *ermC* gene was  
then ligated to the *EcoRV* site of the mutated construct. The fragment containing an  
*ermC* replacement of the *sarR* gene was cloned into the temperature sensitive shuttle

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vector pCL52.1 (57) which was then transformed into RN4220 by electroporation (58) followed by transduction into RN6390 with phage Φ11 as described (15). Transductants were selected at 30°C on erythromycin and tetracycline-containing plates.

5           *S. aureus* RN6390 harboring the recombinant pCL52.1 was grown overnight at 30°C in liquid medium in the presence of erythromycin, diluted 1:1000 in fresh media and propagated at 42°C, a non-permissive temperature for the replication of pCL52.1. This cycle was repeated four times and the cells replicate-plated onto O3GL plates containing erythromycin and erythromycin/tetracycline to select for 10 tetracycline-sensitive but erythromycin-resistant colonies, representing mutants with double-crossovers. The mutations were confirmed by Southern hybridization with *sarR* and *ermC* probes.

15           **Southern Blot Hybridization.** Chromosomal DNA of assorted staphylococcal species was isolated from lysostaphin-treated cells as previously described (15), restriction-digested, resolved in agarose gels and transferred onto a Hybond N+ membrane (Amersham, Arlington Heights, Ill.). Hybridization was performed under high stringency conditions with <sup>32</sup>P-labeled DNA probes as described (15). The blots were subsequently washed and autoradiographed.

20           **Purification of proteins.** The intact 345 bp *sarR* gene was amplified by PCR using RN6390 chromosomal DNA as the template and primers containing flanking restriction sites (*NdeI* and *BamHI*) to facilitate cloning into expression vector pET11b (Novagen). The recombinant plasmid containing the *sarR* gene was transformed to *E. coli* BL21(DE3)pLysS. Enhanced expression of SarR was induced by adding IPTG (isopropyl-1-thio-β-D-galactopyranoside) to a 2 L growing culture (37°C) at an OD<sub>650</sub> 25 of 0.7. After 4 hrs of additional growth, cells were harvested, resuspended in buffer (25mM Tris-Cl, 1mM EDTA, pH 8.0, 100mM NaCl, 10% sucrose and 1mM DTT ), flash-frozen and thawed twice and clarified by centrifugation at 4°C (45,000 rpm for 1 h). After precipitation with 80% ammonium sulfate, the pellets were dissolved in buffer A ( 10mM Tris-Cl, pH 7.5, 1 mM EDTA, 100mM NaCl, 10% glycerol, and 1 30 mM DTT), dialyzed against buffer A, applied to a Resource-Q column in an AKTA purifier (Pharmacia, Piscataway, NJ). The flow-through was re-applied to a Resource-S column and eluted with a NaCl gradient. The fractions were analyzed in

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- a 12% SDS-polyacrylamide gel. Fractions containing the putative SarR protein were pooled, dialyzed against buffer A with 40% glycerol and stored at -80°C. The authenticity of the SarR protein was confirmed by determining the N-15 residues with microsequencing. The concentration of the purified protein was determined with the
- 5 Bio-Rad Protein Assay solution (Bio-Rad Laboratories, Richmond, CA), using BSA as the standard.
- Production of anti-sarR monoclonal antibodies.** Purified SarR protein was used to immunize two BALB/c x SJL/J (F1 cross) mice (100 µg each) to obtain monoclonal antibodies as described (32). The titers of the immune sera were determined by an
- 10 ELISA in which diluted sera were added to microtiter wells precoated with SarR (5 µg/ml) as described by Jones et al. (32). After splenic fusion, antibodies from limited dilutions were screened by an ELISA with immobilized SarR protein. Monoclonal antibodies were then purified from culture supernatants with a protein A-agarose column as described (32).
- 15 **RNA isolation and Northern analysis.** Overnight cultures of *S. aureus* were diluted 1:50 in CYGP broth with appropriate antibiotics and grown to mid-log (OD<sub>650</sub>=0.7 with an 18mm borosilicate glass tube), late-log (OD<sub>650</sub>=1.1) and postexponential phases (OD<sub>650</sub>=1.7). The cells were pelleted and processed with 1 ml of Trizol (Gibco-BRL, Gaithersburg, MD) in combination with 0.1 mm diameter sirconia-silica
- 20 beads in a Fast Prep reciprocating shaker (BIO101, San Diego, CA) as described (12). Ten micrograms of total cellular RNA from each sample was electrophoresed through a 1.5% agarose-0.66M formaldehyde gel in MOPS running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, pH 7.2). RNA was transferred onto Hybond N<sup>+</sup> membranes (Amersham) under mild alkaline conditions by using a Turbo blotter
- 25 system ( Schleicher and Schuell, Keene, NH) as described by the manufacturer. RNA was fixed to the membrane by baking at 80°C for 1 hr. For detection of specific transcripts, gel purified DNA probes were radiolabeled with <sup>32</sup>P-dCTP by using the random-primed method (Ready-To-Go labeling kit, Pharmacia) and hybridized under aqueous conditions at 65°C. The blots were subsequently washed and
- 30 autoradiographed.

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**Promoter fusion analysis with the *gfp<sub>uvr</sub>* reporter gene.** To confirm the effect of the *sarR* mutation on *sarA* promoter activities, *sarA* promoter fragments (P1, P2, P3 and combined P2-P3-P1) (39) were cloned into shuttle vector pALC1484, which is a derivative of pSK236 containing the recombinant *gfp<sub>uvr</sub>* gene. The *gfp<sub>uvr</sub>* gene was 5 constructed by introducing a S65T mutation into *gfp<sub>uvr</sub>* (Clontech, Palo Alto, CA), thereby facilitating a shift in the excitation maxima from 395 to 488nm (26). The *sarA* promoter fragments were then cloned into pALC1484, upstream of the *gfp<sub>uvr</sub>* reporter gene. After sequence confirmation, the recombinant pALC1484s were then 10 electroporated into RN4220 and transduced into *S. aureus* strains RN6390 and its isogenic *sarR* mutant (15).

The activities of *sarA* promoter fragments linked to the *gfp<sub>uvr</sub>* reporter gene in RN6390 and its isogenic *sarR* mutant were assayed by flow cytometry. Bacterial cell suspensions obtained at different parts of the growth cycle were analyzed in a FACScan (Becton Dickinson, Franklin Lakes, NJ). After filtering 15 bacterial samples through a 5 m $\mu$  filter to remove large aggregates, bacteria were detected by side scatter as described by Russo-Marie et al. (56). Fluorescence and side scatter data were collected with logarithmic amplifiers. The fluorescence data were reported in fluorescence units as specified by the instrument (FACScan).

To obtain more quantitative fluorescence data, each of the above 20 *gfp<sub>uvr</sub>* reporter constructs was diluted 1:100 from overnight cultures into fresh CYGP medium and, beginning at the second hour, sampled hourly (200  $\mu$ l) for 10 h to encompass the growth cycle from log to stationary phases. The samples were analyzed for total fluorescence and optical densities (605nm) in microtiter wells in a multi-purpose fluorescence spectrophotometer (FL600, BioTek Instrument, Winooski, 25 VT). The fluorescence units and optical densities were given as reported by the instrument. The background was ~200-300 fluorescence units, with variations of less than 100 units between duplicate samples.

**Cell extracts preparation and Western analysis.** Cell-free extracts from midlog, latelog and early stationary phases (representing OD<sub>650</sub> of 0.7, 1.1 and 1.7, 30 respectively, in an 18 mm borosilicate tube) were prepared from RN6390, the isogenic *sarR* mutant and other staphylococcal strains. Cells were grown in CYGP

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broth (50 ml) supplemented with the appropriate antibiotics. After pelleting, the cells were resuspended in 0.5 ml of TEG buffer (25 mM Tris-HCl, 5mM EGTA; pH 8.0) and cell extracts were prepared from lysostaphin-treated cells as described by Mahmood and Khan (37).

5        Equivalent amounts of cellular proteins were separated in 12% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto nitrocellulose membranes as described (61). The blots were incubated at RT with 1:1000 or 1:2000 dilution of anti-SarR or anti-SarA monoclonal antibodies for 3 h followed by another hour of incubation with a 1:10,000 dilution of goat anti-mouse alkaline phosphatase  
10      conjugate (Jackson ImmunoResearch, West Grove, PA). Immunoreactive bands were detected as described by Blake et al. (3). SeaBlue prestained protein standards (Novex, San Diego, CA) were used for molecular weight estimations.

**Gel shift analysis and DNaseI footprinting.** Gel shift assays were performed to determine the interaction of purified SarR with *sarA* promoter fragments. DNA  
15      fragments were end-labeled with  $^{32}\text{P}$  ATP by using polynucleotide kinase. Labeled DNA fragments were incubated at RT for 20 min with the indicated amounts of purified protein in 25  $\mu\text{l}$  of binding buffer (25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 75 mM NaCl, 1mM DTT, 5% glycerol) containing 0.5  $\mu\text{g}$  of calf thymus DNA. The reaction mixtures were analyzed by nondenaturing polyacrylamide gel electrophoresis  
20      as described (19). The band shifts were detected by exposing dried gels to film.

Footprinting assays with linear DNA template and DNase I were performed using a modification of the method previously described (21). A 49-bp fragment upstream of the *sarA* P2 promoter region (39) was cloned into the *Bam*HI site of pUC18, yielding pALC926. A 109-bp *Eco*RI/*Hind*III fragment from pALC926  
25      was gel-purified and end-labeled with  $^{32}\text{P}$ . PCR fragments containing *sarA* P1 (nt 531-859 and nt 620-859) and P3 (nt 364-525) promoter regions were also used in footprinting reactions. To label these PCR products, only one of the primers was end-labeled with  $^{32}\text{P}$  in the amplification reactions, yielding PCR products labeled at one end. For the assay, the binding reactions were carried out in a 100  $\mu\text{l}$  reaction  
30      volume containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 2 mM DTT, 10  $\mu\text{g}$  of BSA, 0.4  $\mu\text{g}$  of calf thymus DNA, template DNA and varying amounts of the SarR protein at RT for 30 min. DNaseI (0.02 unit)

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(Boehringer Mannheim, Indianapolis, IN) was added and allowed to incubate for 1 min at RT. The reaction mixtures were then extracted with phenol/chloroform. DNA was ethanol-precipitated, resuspended in loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.025% (w/v) xylene cyanol FF and 0.025% (w/v) bromophenol blue) 5 and analyzed on a 6% denaturing polyacrylamide sequencing gel. The positions of the protected regions were identified by comparing the footprint with the A+G sequencing ladder of the same fragment (38).

**Results:** The *sarA* promoters are differentially expressed during the growth cycle, with P1 and P2 promoters being most active during the exponential phase and the P3 10 promoter activated postexponentially (39). Because of the complexity in promoter activation and the ensuing expression of *sarA*, the promoter region upstream of the *sarA* gene may serve as a binding site for one or more *trans* acting factors (2,39). Taking advantage of a P2 promoter sequence (2) that shares homology with a region upstream of the *sar* P1 promoter (Fig. 1A), a DNA-specific column, containing the 15 49-bp P2 promoter sequence, was used to partially purify a ~12kDa protein with binding properties to *sarA* promoter fragments (39). In the schematic shown in Fig. 1A, the positions of the transcription starts (-146, -409 and -711 bp upstream of the translation start) for P1, P3 and P2 promoters are depicted according to published sequence (2). The P1, P3 and P2 transcripts have previously been designated as *sarA* 20 P1, *sarA* P3 and *sarA* P2 transcripts. The 49-bp sequence outlined was used to construct a DNA-specific column as described (27). The relative positions of the *sarA* promoter fragments used in gel shift and footprinting studies are indicated (filled boxes) while the promoter fragments for the GFP transcriptional fusion assays are marked as empty boxes.

25 To further characterize this protein and to investigate its regulatory function, the *sarR* gene product was cloned and characterized using biochemical, immunological and genetic approaches.

**Cloning and sequence analysis of the *sarR* gene.** To clone the gene encoding SarR, we blotted the ~12 kDa protein onto a PVDF membrane for N-terminal sequencing. 30 The first 14 amino acids were X(K)IND(I)NDLVNA(S/T)F, (Seq. ID NO.: 8) with X being an unknown residue while those residues in parenthesis carried a putative assignment. In searching the databank of the partially released *S. aureus* genome

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([www.tiger.org](http://www.tiger.org)), we obtained a partial ORF of 47 amino acid sequence that corresponds to the N-terminal sequence of the ~12 kDa protein. By using two degenerate oligonucleotides of 30-nt each, a 141-bp fragment was amplified to probe a chromosomal digest of *S. aureus* strain RN6390, thus allowing identification of a ~4 kb *Cla*I hybridizing fragment. A plasmid DNA library containing ~3-5 kb *Cla*I fragments constructed in pACYC177 (26) was then screened with the 141-bp PCR-generated probe. A positive clone (pALC1361) yielding a ~4-kb insert at the *Cla*I site of pACYC177 vector was identified. In determining the sequence of the insert, and comparing the insert sequence with that of the 141-bp probe, the DNA sequence of the putative gene *sarR* was obtained (Fig. 1B) (GenBank accession #AF207701). The predicted SarR protein contains 115 amino acids, with a predominance of charged residues (34%) and a predicted molecular size of 13,689 daltons. The *sarR* gene has a putative Shine Dalgarno sequence (AGGAGTGG) (SEQ. ID NO: 9) lying 7-bp upstream of the translation start, with typical initiation (ATG) and termination codons (TAA). To ascertain the transcription start site and the putative promoter boxes, the 5'- end of the *sarR* transcript was mapped by primer extension, using an internal primer of the non-coding strand positioned near the N-terminus of the *sarR* coding region. The transcription initiation site is located 88-bp upstream of the translation start, thereby allowing identification of the putative -10 and -35 promoter boxes as TAGAAT (SEQ ID. No. 10) and TTACCG (SEQ ID. No. 11), respectively (Fig. 1B).

In searching the GenBank for related proteins, the entire SarR protein shares sequence similarity with SarA (SEQ. ID NO. 3), with a high probability score of 1.8e-7 (Fig. 1C). There were also other SarR-homologs in the *S. aureus* database (University of Oklahoma *S. aureus* genome database). Like SarA, the SarR protein has a deduced basic pI (9.23). The sequence similarity between SarR and SarA is 51%, with 28% identity (Fig.1C). In limiting the homology to specific regions, residues 52 -75 of SarR were found to share homology with residues 54-77 of SarA, which, in turn, has a limited but regional sequence similarity to the DNA binding domain of VirF (residues 175-198), a transcription regulator of virulence gene expression in *Shigella flexneri* (18, 25).

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**Over-expression of SarR and production of monoclonal antibodies:** To obtain a large amount of SarR, the *sarR* gene was cloned into pET11b and the gene product was over-expressed under an IPTG-inducible promoter in *E. coli* BL21. The expression, purification, and the purity of the SarR protein are shown in Fig. 2.

- 5 Equivalent volumes of protein fractions during the purification process was applied to a 12% SDS-polyacrylamide gel. Fig. 2, Lane 1, whole cell lysate of *E. coli* containing pALC1357 (pET11b with the *sarR* gene); Fig. 2, lane 2, supernatant of the cell lysate after clarification by centrifugation; Fig. 2, lane 3, supernatant before 40% ammonium sulfate precipitation; Fig. 2, lane 4, pellet resulting from 40% ammonium sulfate  
10 precipitation; Fig. 2, lane 5, pellet from 80% ammonium sulfate precipitation; Fig. 2, lane 6, fall through of the redissolved 80% ammonium sulfate precipitant as applied to a MonoQ column (Pharmacia); Fig. 2, lane 7, fall through from the MonoS column (Pharmacia); Fig. 2, lane 8, NaCl elution from the MonoS column. N-terminal sequencing confirmed the identity of the purified SarR protein. The *SarR* protein was  
15 expressed primarily in the cytosolic fraction (Fig. 2, lane 2). After 80% ammonium sulfate precipitation (Fig. 2, lane 5), the redissolved proteins were dialyzed and applied to an anion exchange column (Resource-Q, Pharmacia), only to be found in the fall-through (Fig. 2, lane 6). The flow-through was then applied to a cation exchange column (Resource-S column, Pharmacia) and eluted with a salt gradient.  
20 Using this purification scheme, SarR was purified to near homogeneity (Fig. 2, lane 8). The authenticity of SarR was confirmed by N-terminal sequencing. The purified SarR was then used to immunize mice for the production of anti-SarR monoclonal antibodies. Three monoclonal antibodies, designated 2A7, 2C7, and 5E4 were obtained. Despite the similarity between SarR and SarA, cross-reactive studies  
25 indicated that anti-SarR monoclonal antibodies only reacted with SarR and not SarA on immunoblots.

**The existence of *sarR* in other staphylococcal strains and other prokaryotes**

- PCR analysis was conducted to determine if the SarR gene is present in other *S. aureus* strains, other staphylococcal species and in other bacteria. This  
30 analysis demonstrates that the *sarR* gene is present in all strains of *S. aureus* as well as in *S. saprophyticus* and *S. haemolyticus*. Blast searches confirm this result.

**Binding of SarR to *sarA* promoter fragments by gel shift and footprinting assays.**

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The interaction between SarR and various *sarA* promoter fragments was examined with gel shift and footprinting assays. Accordingly, purified recombinant SarR from *E. coli* was used in gel shift assays with assorted DNA fragments of the *sarA* promoter region including P2 (nt 1-196, P3 (nt 364-525), and P1 (nt 531-859). The mobility of the labeled DNA fragments became more hindered with increasing concentrations of SarR in gel shift assays (Fig. 4). In the assays, Increasing amounts (30, 60, 100, 150, 200, 250, 300 ng) of purified SarR were applied to the reaction mixtures. In competition assays, 50 and 100 fold excess of unlabeled DNA fragments were added. The unusual laddering pattern of the band shifts was observed with all three *sarA* promoter fragments. One plausible explanation is that each of the *sarA* promoter fragments may contain multiple binding sites. Alternatively, the binding of SarR in multimeric form to a common site or multiple sites within each of the *sar* promoter fragment is plausible. An analysis of the relative binding of SarR and SarA to the *sarA* P1 promoter indicates that the amount of SarA required to completely retard the mobility of 2-5 ng of radiolabeled *sarA* P1 fragment is 10 times more than that of SarR, thus demonstrating the higher avidity of SarR than SarA for the *sarA* P1 promoter fragment.

To determine the binding site of SarR and to verify the specificity of binding to the *sarA* promoter region, DNase I footprinting analysis was performed. To elucidate the SarR binding site, a 109-bp *EcoRI-HindIII* fragment derived from pUC18 containing the 49-bp sequence (39) was end-labeled at the *EcoRI* or *HindIII* sites separately and subjected to DNaseI footprinting with assorted concentrations of SarR. The sequence was deduced from G/A ladder reactions run in parallel following the standard method (26). The following amounts of SarR were applied to the *sarA* P2 and P1 reactions: 30, 60 and 100 ng, With *sar* P1', only lanes containing 30 and 60 ng of SarR protein were shown. The binding sites of SarR on the *sarA* P3 promoter was also mapped: 373 TTACTAAATTAAAAAAATTA 402 (SEA. ID NO. 12) (2). Analysis of the footprint of the plus strand (*EcoRI* site end-labeled) (Fig. 5A) disclosed the protected region (nt 81TAAATTAA 108) (SEQ ID. NO. 13) (2) to be extremely A/T rich (96%), thus implying specific binding of SarR to this region but not to the more GC-rich polylinker region of pUC18, even when higher concentrations of SarR were

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used in the assay. A similar protection site was also found for the minus strand (*Hind*III site end-labeled). In analyzing the SarR protected region, the site was found to consist of a 7-8 sequence (TAAATTAA (SEQ ID. NO. 14), with the last base variable) conserved in both strands (e.g. <sup>101</sup>ATAATT<sub>108</sub> (SEQ ID. NO. 15) being complement of TAAATTAA) and throughout the *sarA* promoter region (39).

The binding of SarR to other *sarA* promoter regions was also determined. It has been shown that an inverted repeat region (nt 553-593) upstream of the *sarA* P1 promoter may play a role in repressing *sarA* P1 transcription (39). Recognizing that SarR binds to a large P1 fragment in gel shift assays (Fig. 4), a footprinting analysis was performed with two different DNA fragments upstream of the *sarA* P1 promoter [329-bp (nt 531-859) and 240-bp (nt 620-859)(2)]. Using <sup>32</sup>P end-labeled sense strand, the SarR-protected region on the 329-bp *sarA* P1 promoter fragment was found to comprise several regions including nt 551 to 553, 556 to 575, 582 to 603 (<sup>586</sup>TAAATTAT<sup>593</sup>) (SEQ ID. NO. 16), 620 to 640 (Fig. 5B). In analyzing the smaller 240-bp P1 fragment, four additional protected regions, downstream of the above binding sites, were uncovered: nt 633 to 640, 643 to 667, 673 to 678, and 687 to 708 (Fig. 5C). Thus, the inverted repeat region (nt 553-593), which has previously been shown to play a putative role in repressing P1 transcription (39), is also part of the SarR binding sites. The SarR binding site on the *sarA* P3 promoter was also uncovered:

373 17 TTACTAAATTAAAAAAATTA 402 (SEQ I.D. NO. 17) (2). In comparing the broad binding sites protected by SarR, a common feature is their highly AT-rich nature. More remarkably, the 7-8 bp conserved sequence (TAAATTAA) (SEQ. ID NO:14) was included within the SarR binding sites in each of the *sarA* promoter fragments (P2, P1 and P3).

**Expression of the *sarR* gene in RN6390 and its isogenic *sarA* and *agr* mutants:**  
During the growth cycle, the major *sarA* gene product such as SarA partially mediates its effect by binding to the *agr* promoter to influence RNAII and RNAIII transcription. To ascertain if the *sarR* gene is modulated by *sarA* or *agr* (i.e. acting downstream of the *sarA* or *agr* regulatory cascade), *sarR* transcription in parental strain RN6390 and its isogenic *agr* and *sarA* mutants was assayed. To ensure that comparable amounts of total cellular RNA were applied to each lane, ribosomal RNA

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bands stained with ethidium bromide were compared among the lanes (Fig. 6A). Ten µg of total cellular RNA was applied to each lane. The *sarR* probe was a 345-bp fragment (nt 208-552). The OD<sub>650nm</sub> of 0.7, 1.1 and 1.7 represent mid log, late log and early stationary phase, respectively, as predicted from the growth cycle. As  
5 displayed in Fig. 6B, the transcription of *sarR* in RN6390 could be detected in mid log phase and was maximally expressed during the postexponential phase. Accounting for minor experimental variations, the observation that *sarR* transcription was not significantly altered in *sarA* and *agr* mutants indicated that *sarA* and *agr* did not regulate *sarR* as one would expect if these regulating loci lie downstream of *sarR*.  
10 This is consistent with the finding (described below) that a mutation in *sarR* affects *sarA* and *agr* transcriptions.

The expression of the SarR protein during the growth cycle by immunoblots was also determined. Using anti-SarR monoclonal antibody 2C7 (1:1000 dilution), an immunoblot of cell-free extracts of RN6390 derived from cells grown to late-log (OD<sub>650</sub>=1.1) and postexponential phases (OD<sub>650</sub>=1.7) was probed.  
15 Employing ~25 µg of cellular proteins in each lane, it was found that the expression of SarR corresponds quite well with the pattern of *sarR* transcription, with SarR detectable at late-log phase, and maximal during the postexponential phase (Fig. 6C). In figure 6C, Each lane contains cell-free extracts (25 µg each) of RN6390 cells  
20 grown to late-log and early stationary phases. Cells at mid-log phase expressed little SarR; as expected, SarR was not detected in the *sarR* mutant ALC1713 (data not shown). The positive control lane contains 0.1 µg of purified SarR protein.

**The expression of *sarA* in a *sarR* mutant:** To demonstrate that the SarR protein likely modulates *sarA* expression by virtue of its binding to the *sarA* promoter region,  
25 a *sarR* deletion mutant was constructed by replacing the *sarR* gene with an *ermC* gene in strain RN6390. Northern analysis confirmed that the transcription of *sarR* was disrupted in *sarR* mutant ALC1713. To analyze the effect of *sarR* on individual *sarA* promoters, P2 (nt 1-180 plus 197-bp upstream), P3 (nt 364-525), P1 (nt 620-859) and the combined (or native) P2-P3-P1 promoters (nt 1-859 plus 197-bp upstream) (2, 39)  
30 upstream of the *gfp<sub>uvr</sub>* reporter gene were cloned in shuttle plasmid pALC1484.

Flow cytometry was used to evaluate promoter activity, demonstrated that the *sarA* P1 and the combined P2-P3-P1 promoters were more active in the *sarR* mutant than the

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parental control. Mean fluorescence was  $5.01 \pm 0.29$  (log scale) in RN6390 vs  $5.84 \pm 0.13$  in *sarR* mutant and  $5.49 \pm 0.21$  in RN6390 vs  $8.44 \pm 0.24$  in the mutant, for P1 and combined promoters, respectively, during the postexponential phase. However, the relative weakness of the *sarA* P3 and P2 promoters as compared with the P1 promoter (~20 to 30 fold less than of P1) (39), coupled with the relative stability of the GFP reporter, rendered flow cytometry less useful to record small variations in GFP expression during the growth cycle among 10,000 organisms gated for this experiment. Not surprisingly, we failed to detect differences in activation of the weaker *sarA* P3 and P2 promoters between the parent and the isogenic *sarR* mutant by flow cytometry (39). More specifically, the level of P2 and P3 activation as detected by flow cytometry was only slightly above backgrounds.

To obtain more quantitative fluorescence data for a larger number of bacterial cells, a multi-function fluorescence spectrophotometer in a microtiter format was used (FL600 from BioTek Instrument, Winooski, VT) to measure optical densities and total fluorescence of samples (200  $\mu$ l) obtained serially during the growth cycle. To minimize the variation in fluorescence attributable to cell density, fluorescence per OD against optical densities was plotted over a 10 h period (extending from log to stationary phase). The data showed that the *sarA* P1-GFP fusion activity in the *sarR* mutant was higher than the parental strain (RN6390) throughout the growth cycle (Fig. 7A). Figure 7A illustrates recombinant shuttle plasmid pALC1484 containing the *sarA* P1 promoter linked to *gfp<sub>uvr</sub>* (excitation maxima 488nm) in strain RN6390 ( $\mu$ ) and its isogenic *sarR* mutant ALC1713. A negative control (RN6390 containing only pALC1484 without any promoter fragment) did not display any significant background fluorescence (~300 fluorescence units as the background, data not shown). Cells were obtained hourly (200  $\mu$ l each in duplicate) during the growth cycle (from the 2<sup>nd</sup> to the 10<sup>th</sup> h after an initial dilution of 1:100 in fresh medium) to obtain fluorescence and optical densities (OD) in the same instrument. The data were presented as the average of reported fluorescence per OD in triplicate samples plotted against the mean of the optical densities. The error bar was too small to be discerned, typically less than 100 fluorescence units. This experiment has been repeated at least thrice. One representative experiment was shown. The early decline in fluorescence for both strains was likely to be attributed to

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the carryover effect of the GFP from the overnight inoculum. Following the decline, the highest level of GFP fusion in the mutant occurred late in the stationary phase (at ~7-10 h after initial culture dilution) (Fig. 7A), at a time when the *sarR* transcript level and the expression of SarR in the parent were highest (Fig. 6B and 6C). The 5 level of GFP fusion for the *sarA* P1 promoter in the parental strain declined after the initial dilution, but higher than the background (background fluorescence ~300 units), during the growth cycle. This finding is attributable to a steady decrease in P1 promoter activation (per bacterial cell) in the parental strain as the cell cycle progressed. Additionally, a lack of contribution from upstream promoters (i.e. P3 and 10 P2) to modulate P1 activity in this promoter fragment may conceivably play a role. Similar studies were also conducted for the combined or native *sarA* P2-P3-P1 promoter linked to the *gfp<sub>uvr</sub>* reporter. In this instance, the combined promoter activity in the *sarR* mutant was also higher than that of the parent. Figure 7B illustrates a plot similar to Figure 7A except that the combined *sarA* P2-P3-P1 15 promoter fragment was used in place of the P1 promoter in the recombinant pALC1484 containing the *gfp<sub>uvr</sub>* reporter gene. Similar assays were also performed with the individual *sar* P2 and P3 promoters linked to the *gfp<sub>uvr</sub>* reporter in the isogenic pair, but failed to detect any differences in GFP<sub>uvr</sub> expression between the parental strain and the *sarR* mutant. However, the level of fluorescence associated 20 with individual P2 and P3 promoters was very low and only slightly above backgrounds. As with the P1 promoter, the level of activity for the combined promoter decreased after initial culture dilution for both strains and then increased during the postexponential phase. Of interest, the increase in combined promoter activity with growth in the parental strain suggests that the sequence element 25 upstream of P1 may have contributed to the overall increase in combined promoter activity during the postexponential phase. However, no differences in fluorescence for the P2 or the P3 promoter GFP fusions between the *sarR* mutant and its isogenic parent were detected. Notably, the fluorescence of the P2 and P3 promoters was only slightly above background. Thus, this shows that the fluorescent assays may not be 30 sensitive enough to detect subtle differences in P2 and P3 promoter activities. To demonstrate that an intact *sarR* gene has a negative impact on the expression of SarA, the major *sar* regulatory molecule, cell-free extracts of the isogenic *sarR* strains

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5 during various stages of the growth cycle were obtained. Using cell extracts (25 µg of proteins each) of the *sarR* mutant obtained at different phases of the growth cycle, an immunoblot with anti-SarA monoclonal antibody 1D1 (20) was probed. As shown in Fig. 8A, the *sarR* mutant expressed higher levels of SarA protein than the isogenic parent at optical densities representing mid-log, late-log and stationary phases. Fig. 8A shows an immunoblot of the cell extracts (5 µg of protein each) of RN6390 and the *sarR* mutant (harvested at midlog, late-log and stationary phase) probed with anti-SarA monoclonal antibody 1D1 at 1:2000 dilution. The positive control lane contains 0.5 µg of purified SarA. Similar results were obtained with 25 µg of protein per lane. Notably, in both the parental and the mutant strains, SarA expression was maximal during the late-log phase and tapered toward the stationary phase. This shows that the reduction in SarA expression in the parental strain during the stationary phase is explained in part by increased proteolytic activity and hence processing of SarA in stationary cells (39, 51). Additional immunoblots with increased amounts of 10 cell extracts (at 25 µg each) from mid-log, late-log and stationary phases also confirmed higher expression of *sarA* in the *sarR* mutant as compared to the parental strain. Taken together, these data demonstrate that SarR is a DNA binding protein 15 that binds to the *sarA* promoter to down-regulate *sarA* expression.

20 The level of SarA has been shown to correlate with the extent of *agr* activation (20). Northern analysis of *sarR* mutant ALC1713 with an *agrA* (RNAII) probe confirmed a higher level of RNAII expression as compared with the parental control (Fig. 8B). Collectively, these data demonstrate that SarR down-regulates *sarA* expression, by binding to the *sarA* promoter to down-modulate *sarA* P1 transcription. The end result is the modulation of target genes (e.g. *agr*) downstream of the *sarA* 25 regulatory cascade.

**Protein expression, purification and crystallization:** The intact 345-bp *sarR* gene was amplified by PCR using chromosomal DNA from *S. aureus* strain RN6390 as the template and primers containing flanking restriction sites (*Nde*I and *Bam*HI) to facilitate cloning into an expression vector pMAL-c2 (BioLabs, Beverly, MA) modified by truncating 21 residues from the linker region that connects SarR and MBP. The recombinant plasmid containing the *sarR* gene was transformed to *E. coli* BL21(DE3)pLysS. Enhanced expression of *sarR*-MBP fusion was induced by 30

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adding IPTG (isopropyl-1-thio-β-D-galactopyranoside) to a 4 L growing culture (37°C) at an OD<sub>650</sub> of 0.7. After 4 hrs of additional growth, cells were harvested, resuspended in buffer (50mM Tris-HCl, 1mM EDTA, pH 7.4, 300mM NaCl, 5% glycerol and 1mM DTT) and subjected to cell lysis through a continuous-flow French press. After a 20,000xg spin, the soluble fraction was loaded onto an amylose resin affinity column (10ml) and the SarR-MBP fusion protein was eluted with 10 mM maltose. The protein was loaded onto a MonoQ (Pharmacia) ion-exchange column. After elution with a NaCl gradient (0.1 to 0.5 M) the fraction containing the protein was found to be homogeneous as determined by a Coomassie stained SDS-polyacrylamide gel. The concentration of the purified protein was determined with the Bio-Rad Protein Assay solution (Bio-Rad Laboratories, Richmond, CA), using BSA as the standard. The SarR-MBP fusion protein (15mg/ml) was crystallized by vapor diffusion against a solution of 5 mM β-mercaptoethanol, 100 mM Na acetate, 100 mM NaCl, pH 4.6, and 18-22% PEG monomethyl ether 2000. For cryo-crystallography, crystals were soaked in steps of increasing glycerol concentration (5% each step every 30 min) and finally into 20% glycerol before flash-freezing.

**Structure Determination and Refinement:** Data were collected on a Rigaku R-axis IV system and beamline 5.0.2 at ALS. Data processing was performed with DENZO and SCALEPACK (50). The initial phases were obtained by a molecular replacement solution using the available MBP structure (53) and program AMoRe (45). 2Fo-Fc and Fo-Fc electron density maps were calculated by CNS (6). Map interpretation and model building were done using the program O (33). The map was improved by cycles of refinement using CNS with NCS constraints. A final refinement was performed with relaxed NCS-restraints as shown in Table 2 below. The final model contains residues 1-115 for molecule one of SarR, residues 1-115 for molecule two of *sarR* (Fig. 9B), residues 1-372 for both MBP molecules (Fig. 9A), two maltose molecules, and 190 water molecules. Stereochemical values are all within or better than the expected ranges for a 2.3 Å structure, as determined using PROCHECK (35).

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**Table 2** Summary of Crystallographic Analysis

## Diffraction Data and MR statistics

Crystal <sup>a</sup>	Resolution (Å)	R <sub>merge</sub> <sup>b</sup> (%)	Reflections Measured/Unique	Completeness (%)	Solutions <sup>c</sup>	C.O. <sup>d</sup>	R <sup>e</sup>
Native I	2.8	4.3	62396/20967	78.0	2	47.2	
Native II	2.3	2.7	77545/37403	96.5			46.6
<hr/>							
Refinement (Native II)							
Resolution (Å)	20-4.58	3.65	3.19	2.90	2.69	2.53	2.40
No. reflections	5696	5656	5512	5198	4788	4180	3454
R-factor <sup>e</sup>	15.27	20.16	29.30	33.14	36.95	38.04	38.86
Free R-factor <sup>d</sup>	21.94	24.45	32.45	38.65	39.36	38.79	35.91
r.m.s. Deviations; Bonds, 0.009 Å; Angles, 1.4°							

<sup>a</sup> Crystal spacegroup P1; a = 64.7 Å, b = 70.6 Å, c = 75.5 Å, α = 65.7°, β = 67.2°, γ = 69.6°, two sarR-MBP monomers/unit cell<sup>b</sup> R<sub>merge</sub> = 100 ×  $\sum_j |I_j - \langle I \rangle| / \sum_j I_j$  with Bijvoet pairs treated as equivalent<sup>c</sup> R-factor =  $\sum |F_{\text{obs}} - F_{\text{calc}}| / \sum F_{\text{obs}}$  for all amplitudes with  $F/\sigma(F) \geq 2$  measured in the indicated resolution bin<sup>d</sup> Free R-factor was calculated with 5% of the data in each bin.<sup>e</sup> Molecular replacement solutions, C.O. was correlation coefficients, and R, are defined in Amore (18)

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**Overall Structure:** The structure of SarR-MBP fusion protein shows that the SarR dimer is located at the top of the two individual MBP molecules, connected by two flexible loop regions between SarR and MBP. The positions of the MBP molecules suggest that they did not influence the structure of SarR dimer (Fig. 9A). The overall  
5 structure of the SarR monomer consists of five  $\alpha$  helices, three short  $\beta$  strands, and several loops (Fig 9B). The  $\alpha$ 1 helix extends out from the remaining molecule, forming a 'L' shaped like structure with a stretch formed from the N-terminal residues of the protein. The  $\alpha$ 2 helix follows a 7- residue loop from  $\alpha$ 1, and is almost perpendicular to  $\alpha$ 1 ( $85^\circ$ ). The three  $\beta$  strands,  $\beta$ 1,  $\beta$ 2, and  $\beta$ 3, form an anti-parallel  
10 bundle, which is slightly twisted.  $\alpha$ 5 follows immediately after  $\beta$ 3. Between  $\beta$ 1 and  $\beta$ 2 is a long flexible region (residues 51-79), which has poor electron density in the initial 2Fo-Fc and Fo-Fc maps, containing two helices,  $\alpha$ 3 (residue 51-56) and  $\alpha$ 4 (residue 63-75) respectively, and a short turn (residue 56-58). These three elements build up a typical helix-turn-helix structural module existing in DNA-binding  
15 proteins. Homology alignment of the SarR structure with all available structures shows that the SarR monomer is homologous to winged helix proteins (23), such as transcription regulatory protein mota fragment (PDB code 1bjb) with Z score of 8.0, transcriptional repressor smtb activation domain (PDB code 1smt) with Z score of 7.2. Compared to winged helix proteins, "W2" is replaced by a helix ( $\alpha$ 5) and the "W1"  
20 extends much further in the SarR monomer (Fig. 9B). The above data shows that SarR and its family of proteins are new members of the classic winged helix protein family.

Two L-like structures of  $\alpha$ 1 and the stretch of the N-termini come together forming a dimer that has an elongated, slightly bent structure with overall  
25 dimensions of  $71\text{\AA} \times 37\text{\AA} \times 34\text{\AA}$  (Figs. 10A and 10B). The monomers are related to each other by a non-crystallographic local two-fold axis. On the concave side and middle of the SarR dimer, there is a canyon like structure with length of  $\sim 35\text{\AA}$ , a width of  $\sim 25\text{\AA}$ , and a depth of  $\sim 10\text{\AA}$  (Figs. 10A and 10B). The canyon is formed by a part of  $\alpha$ 1, the loop that connects  $\alpha$ 1 and  $\alpha$ 2 and a part of  $\alpha$ 2 as its bed, and with  $\alpha$ 4  
30 from both monomers acting as the two banks (Figs. 10A and 10B). On the convex side, all four termini (C and N-termini of both molecules) form a flat platform with

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- the N-terminus of one monomer adjacent to the C-terminus of the other. This feature suggests that SarS, a 250 residue protein homologous to SarA (23), functions as a heterodimer-like monomer since it contains two sections, highly similar to the SarR module, each 125 residues long (17). The entire dimer can be described as three
- 5 individual subdomain structures (Fig. 10A). Three beta strands plus  $\alpha$ 3 and  $\alpha$ 4 from each molecule form subdomain 1 and subdomain 2, respectively. Those two subdomains were poorly defined in the initial electron density map, which also was reflected by their high temperature factors compared to the average (89 via 69 ) in the final model, and can be expected to have high mobility in the molecule in solution.
- 10 Subdomain 3, consisting of the major alpha helices  $\alpha$ 1,  $\alpha$ 2, and  $\alpha$ 5 from both monomers, is relatively rigid due to restraints of hydrophobic interactions. These helices in subdomain 3 are arranged in such a manner that the entire subdomain looks like a twisted letter Z with the relatively flexible subdomain 1 and subdomain 2 covering its ends. The Z-shaped scaffold, a unique structural feature for SarA family,
- 15 represents a new functional protein fold (Figs. 2A and 2B).

**Dimerization Interfaces:** Several lines of evidence suggest that, with the exception of SarS, the active form of the SarA family of proteins is a homodimer. First, there is strong evidence showing that SarA exists as homodimers *in vitro* as well as *in vivo* (60). Second, upon mixing partially unfolded (4M urea) full length SarR protein and

20 SarR-MBP fusion, a heterodimer product containing one copy each of SarR and SarR-MBP fusion could be separated by gel-filtration chromatography. Third, crystals of SarR-MBP fusion protein were used to solve the SarR structure. The SarR protein exists as a dimer even in the MBP fusion form and indicating that the dimerization form is the active form existing *in vivo*. Fourth, based upon the homodimer SarR

25 structure, the interactions between the two monomers are quite extensive, with most residues involved in hydrophobic interactions (Fig. 9A and 11). Residues Leu10, Ile7, Ile 4 from one monomer and residues Phe20, Ile35, Leu 109 and Ile 113 from the other monomer form two hydrophobic cores. The dimer interface buries  $\sim$ 1,500  $\text{\AA}^2$  of solvent-accessible surface area (1.4  $\text{\AA}$  probe, 25) per monomer. The hydrophobic

30 feature of SarR dimerization, indicates that the dimer of SarR in the fusion protein is also the functional dimer *in vivo*. Furthermore, the L shape like structure of  $\alpha$ 1 and the stretch of the N-terminus of SarR physically block the dissociation of one

monomer from its partner. This explains the need for extremely harsh conditions (e.g., 4M urea) in order to disrupt the dimerization of individual monomers. Fifth, deletion analysis showed that mutated *sarA*, with a 15-residue truncation at its N-terminus, exists as monomers *in vitro* (in solution or crystal packing forms). Finally,  
5 as shown by the sequence alignment of SarA, SarR, and SarS, most residues involved in the dimerization process are highly conserved (Fig. 11).

**DNA Binding and Bending:** The DNA binding domain of SarR is conserved in the SarA protein family. The binding domains include the helix turn helix motif and the wing of the SarR molecule. SarA and SarR proteins contain a high percentage of the  
10 residue Lys (39). Remarkably, most of the Lys residues are highly conserved between these two proteins (Fig. 11). It was predicted that most of these Lys residues are involved in DNA binding (39). Although the Lys residues are distributed throughout the entire primary sequence, in the 3D structure, most Lys residues and some Arg residues are located primarily on one surface of the SarR dimer (i.e., the  
15 concave side). The electrostatic potential on this surface of the SarR dimer, calculated by the Grasp program (46), revealed a positively charged track on this side (Fig. 12A). Additionally, the two winged helix motifs (domain 1 plus  $\alpha$ 2 from one monomer and domain 2 plus  $\alpha$ 2 from another monomer) are located on this side. Accordingly, this side is apparently the site for DNA binding.

20 Using the program Dali to search for proteins with a structure similar to *SarR*, every structure with a Z score higher than 4 was manually examined. It was determined that the spatial arrangement of the two SarR helix-turn-helix modules is quite similar to those in the catabolite activating protein (CAP) (33, PDB code 1cgp with Z score of 4.7). Superposing the SarR winged helix motif 1 and 2 with the  
25 corresponding domains from CAP dimer, the root mean square deviation (rmsd) of the  $\alpha$ -carbon backbone for motif 1 is 1.8 Å, and 2.1 Å for motif 2 (Fig. 13A). Interestingly, some of the residues involved in DNA binding and bending in the CAP dimer, which interact with the major grooves, are conserved in the SarR dimer and in other members of the Sar family (Fig. 11). For example, SarR charged residues that  
30 are predicted to interact with phosphate groups on the DNA backbone, are possibly Lys 52, Lys 56, Lys 71, and Arg 82. Lys 52 and Arg 82 are absolutely conserved in SarR and in the SarA family of proteins. Charged residues predicted to contact DNA

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\ bases are Lys 61 and Lys 67. Lys 61 is also absolutely conserved. Therefore, this shows that the SarR dimer and other SarA family members have similar protein-DNA interaction as the CAP dimer.

The interaction causes the DNA to bend at two points by ~90° (59).

- 5 The longest direct distance for the CAP and SarR dimer surface is ~ 71 Å (Fig. 12), which can hold a stretch of bent DNA with ~27 base pair nucleotides that has length of ~92 Å for a normal B-form (59). This is consistent with the experimental DNA footprinting data, showing that ~29 nucleotides from the *sarA* promoter region were involved in binding to SarR (21). This predicted bending of the DNA when SarR  
10 binds to DNA, may reflect a regulatory mechanism for the SarA protein family in controlling target gene transcription.

The SarR dimer was superimposed on the CAP-DNA complex to construct a model for a SarR-DNA complex (Figs. 13B and C). This model suggests that in addition to interactions of the  $\alpha$ 4 helix with the DNA major groove, SarR  
15 makes contacts with the DNA minor groove. The loop region between  $\beta$ 2 and  $\beta$ 3 and part of the two beta strands (W1, a  $\beta$ -hairpin) should be quite flexible in the free *SarR* structure. Only slight adjustments of their conformations are required to position them to interact intensively with the minor groove of the DNA. Several residues that could be involved in the interactions are highly conserved: Asp 86, Glu 87, and Arg  
20 88 with the side chain of Arg 88 interacting with the DNA phosphate backbone and the side chains of Asp 86 and Glu 87 interacting with bases (Figs. 13B and C). This loop is too short in CAP and other winged helix proteins to have this minor groove binding function (23). One new member of one of the classes of winged helix proteins (RFX) does make DNA minor groove contacts, but in this case, the wing  
25 contacts the major groove and the helix contacted the minor groove (24). Therefore, the predicted SarR type of wing-minor groove interaction appears to be a unique feature of the SarA family of proteins establishing them as a third class of the winged helix family (23,24).

The two subdomains involved in the DNA binding and bending in  
30 CAP have different conformation at the absence of DNA, but are identical in the complex structure (23). The corresponding subdomains (1 and 2) in SarR are almost identical, but most of side chains are poorly defined. Those two subdomains should

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have high mobility in the absence of DNA just as these are in the CAP protein. The mobility can be reflected by their high temperature factors (Fig 9A).

**Regulation Mechanism:** One class of transcription-activating proteins bears two structural motifs, namely a DNA binding and an activation domains (52). The SarR

- 5 protein was initially defined as a transcriptional repressor protein that binds the *sarA* promoter region (39), thus leading to reduced transcription from the *sarA* P1 promoter. As stated previously, the *sarA* P1 promoter is the predominant promoter in the *sarA* regulatory system. Transcriptional fusion studies indicate that the *sarA* locus is auto-regulatory (21), possibly mediated by the binding of SarA to its own  
10 promoter. The binding affinity of SarR to a *sarA* promoter fragment is higher than its SarA counterpart (40), consistent with the idea that an activation motif might be present on the SarA protein but not on the SarR protein, and that SarR may repress by a simple competitive displacement mechanism. A second possibility is that SarA and SarR may form a hetero-dimer to interfere with the function of the SarA homo-dimer.  
15 Due to the conservation of residues involved in the dimerization, this could happen *in vivo*. Finally, SarR may function similarly to the bacteriophage lambda repressor (which also has a helix-turn-helix DNA binding motif). In this case, a slight DNA binding site difference (one base pair shift) could turn an activator to a repressor by affecting the RNA polymerase binding (7).

- 20 The structure of the SarR protein, combined with the sequence alignment of additional SarA family members (Fig. 11), shows that the regulatory mechanism is complicated. Residues 7, 8, and 11 appear to be important residues in the activation domain. When compared to the CAP, there is no isolated activation domain in the SarR dimeric structure, a calculation of the surface electronic potential  
25 revealed two negatively charged patches on the convex side of the SarR dimer (Fig. 12B). These patches include residues Asp6 and Asp9 from molecule 1, Glu108, Glu110, and Glu111 from molecule 2. Many transcription regulators work by binding DNA and then interacting with a component of the RNA polymerase machinery (52). For example, CAP regulates downstream protein expression mostly through its  
30 interactions with the C-terminal domains of alpha subunit of RNA polymerase (28). These acidic patches on the surface of SarR may represent activation motifs that allow SarR to regulate gene expression in a similar way. Since Glu108, Glu110, and

- 40 -

Glu111 are not conserved among the SarA family (Fig. 11), this activity may have a different specificity among the family members. In this regard, we have determined that SarR, besides interacting with the *sarA* promoter, also directly binds to the *hla* promoter (e.g. alpha hemolysin gene), thus bypassing the effect of *sarA* in controlling  
5 target genes.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the  
10 invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

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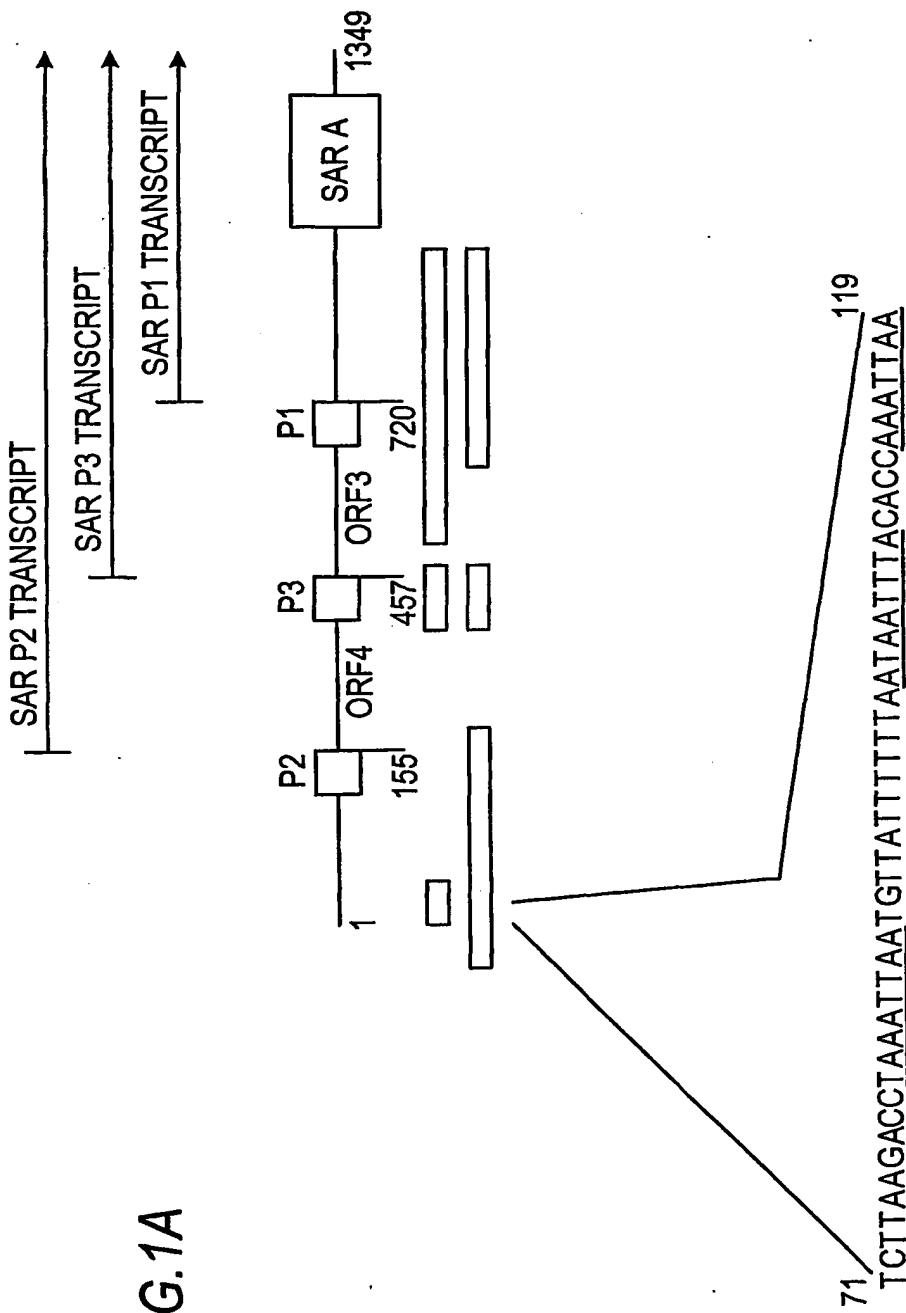
What is claimed is:

1. A nucleic acid sequence which regulates the expression of virulence determinants in gram positive bacteria.
2. The nucleic acid sequence of claim 1, wherein the bacteria is a *Staphylococcus* species.
3. The nucleic acid sequence of claim 2, wherein the bacterium is *Staphylococcus aureus*.
4. The nucleic acid sequence of claim 1 comprising the sequence as essentially set forth in SEQ ID NO: 1.
- 10 5. A vector comprising the nucleic acid sequence of claim 4.
6. A host cell comprising the vector of claim 5.
7. A method for identifying putative agents which inhibit growth and infectivity of bacteria comprising identifying agents which enhance the expression of the nucleic acid sequence of claim 1 or the activity of a polypeptide encoded thereby.
- 15 8. The method of claim 7 wherein the bacterium is *Staphylococcus* species.
9. The method of claim 8 wherein the bacterium is *Staphylococcus aureus*.
10. A method of inhibiting growth and infectivity of bacteria comprising contacting the bacteria with an agent which enhances the expression of the nucleic acid sequence of claim 1 or the activity of a polypeptide encoded thereby.
- 20 11. The method of claim 10 wherein the bacterium is a *Staphylococcus* species.
12. The method of claim 10 wherein the bacterium is *Staphylococcus aureus*.
13. A pharmaceutical composition for use as an anti-bacterial agent comprising an agent which enhances the expression of the nucleic acid sequence of claim 1 or the activity of a polypeptide encoded thereby and a pharmaceutically acceptable vehicle.
- 25 14. The composition of claim 13 which is an anti-bacterial agent against a *Staphylococcus* species.
15. The composition of claim 13 wherein the bacterium is *Staphylococcus aureus*.
16. An isolated polypeptide which regulates the expression of virulence determinants in gram positive bacteria.
- 30 17. A polypeptide encoded by the nucleic acid sequence essentially as set forth in SEQ ID NO: 2.

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18. The isolated polypeptide of claim 16 wherein the bacterium is *Staphylococcus* species.
19. The isolated polypeptide of claim 18 wherein the bacterium is *Staphylococcus aureus*.
- 5 20. A kit for identifying the presence of a sarR gene or sarR gene product comprising a means for analyzing a biological sample for the presence of the sarR gene or the sarR gene product.
21. A method of treating a mammal suffering from or susceptible to a gram positive bacterial infection comprising administering a compound capable of selective occupation of a sarA promoter receptor.
- 10 22. A method of screening for lead compounds which inhibit the expression of virulence determinants in gram positive bacteria comprising identifying chemical entities having structural similarities to the SarR protein sufficient to allow said chemical entities to form heterodimers with SarA protein.
- 15 23. A method of screening for lead compounds which inhibit the expression of virulence determinants in gram positive bacteria comprising identifying chemical entities having structural similarities to the SarR protein sufficient to allow said chemical entities to bind sarA promoter receptors.
- 20 24. A pharmaceutical composition comprising a compound identified by the screening method of claim 22 or 23.
- 25 25. A pharmaceutical composition comprising a compound identified by the screening method of claim 22 or 23, wherein said composition is capable of inhibiting the expression of sarA.
26. A pharmaceutical composition for the treatment of gram positive bacteremia comprising a SarR agonist.
27. A pharmaceutical composition comprising a compound that binds to the P1 promoter region of a *sarA* gene.

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1 GTTTCAAAA TCGGTGGAGG TGCATGAAAA AGTTATTGGG CATTTTTGA AAATAAAAAA  
 61 ATATCAATAA GTTGGAGTC TTACCGAATT -35 TTTATACTTA TTTGTTIAGA -10 ATGAACCTTA  
 121 TAACATAGTT GGATAGAGTT TTGATTAA TACATTAAT GTGAACCTTG CTACAACAAG  
 181 ATGTGCATCA GAAGGAGTGG TTTATAATG --- SarR - TAA [SEQ. I.D. NO. 1]  
 RIBOSOMAL BINDING SITA

FIG. 1B

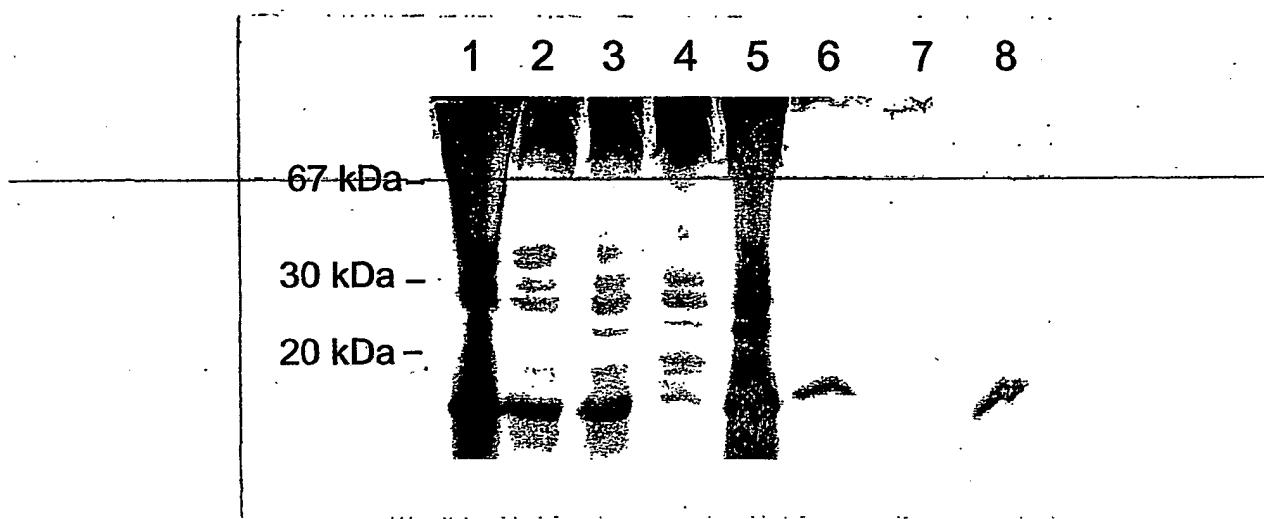
SarR M- SKI NDI NDLVNATF QVKKF FRDTKKFKNLNYEEI YILNHI LRSESNEISSKEIAKCS  
 SarA MAITKI NDCAFELLSMWTYADKLKSLIKKEFSISFEEFAVLTYISENKEKEYYLKDIINHL  
 10 20 30 40 50

SarR EFKPYYLTAKLQKLQLSQKRILQDERTVIVYVT-----DTLKANIHKLISELEE  
 SarA NYKQPQVVKAVKILSQEVDYFDKKRNEHDERTVILVNAQQRKKI ESLLSRVNKRI TEANN  
 60 70 80 90 100 110

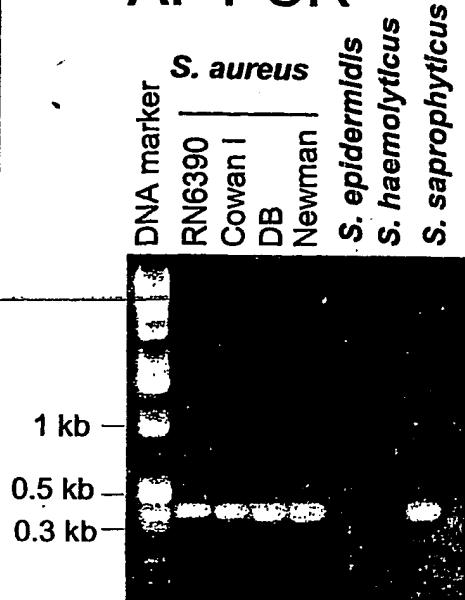
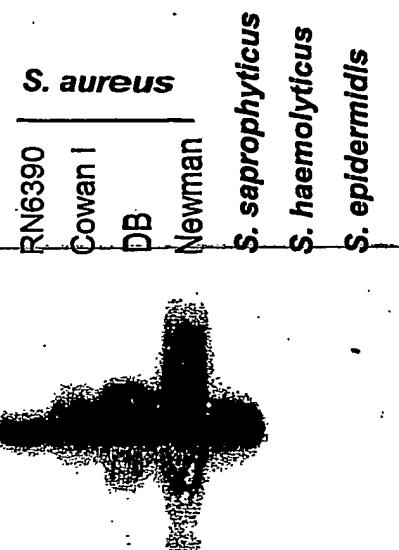
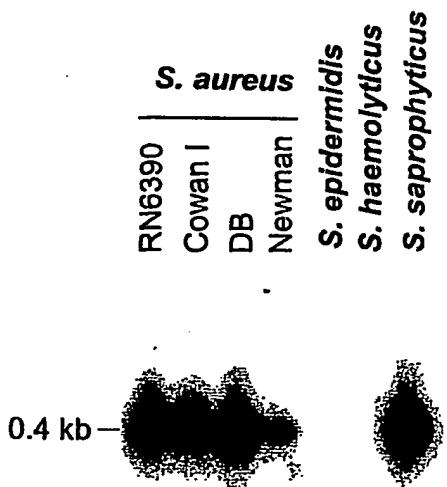
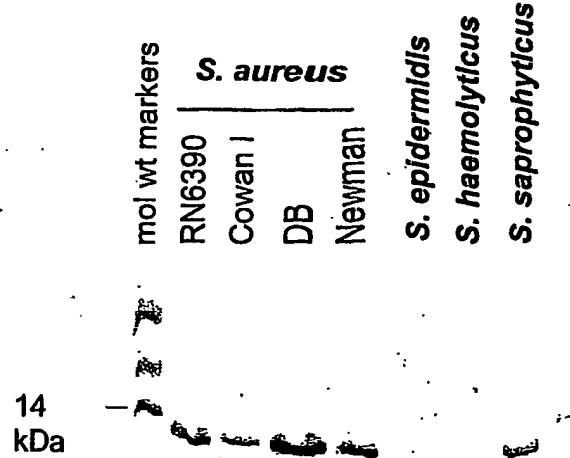
SarR = SEQ I.D. NO.2  
 SarA = SEQ I.D. NO.3

FIG. 1C

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**FIG. 2**

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**FIG. 3A****A. PCR****FIG. 3B****B. Southern****FIG. 3C****C. Northern****FIG. 3D****D. Immunoblot**

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FIG. 4A

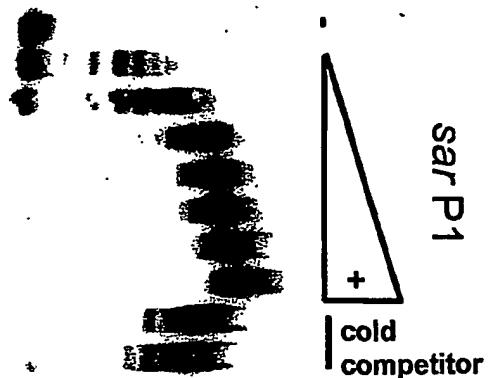


FIG. 4B

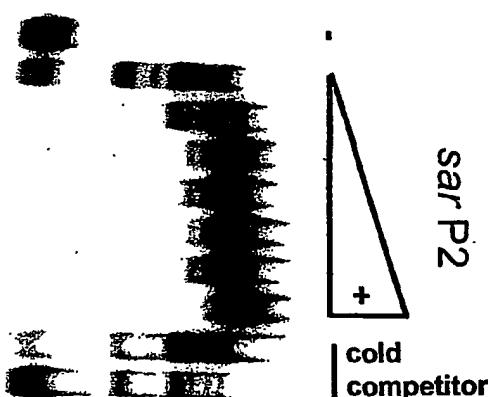
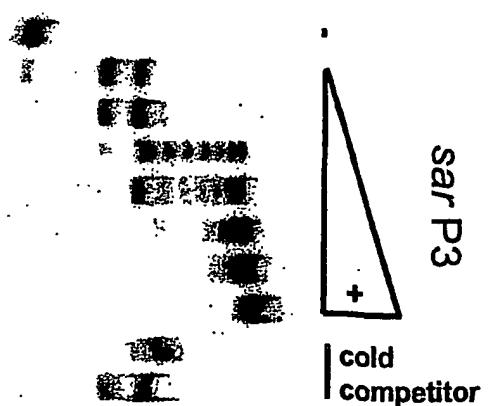
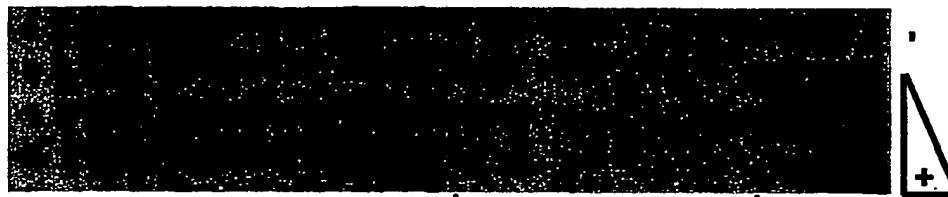


FIG. 4C

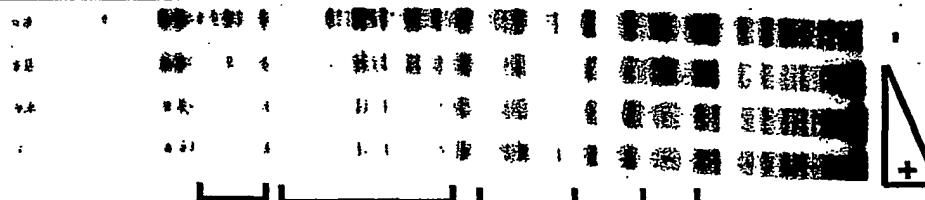


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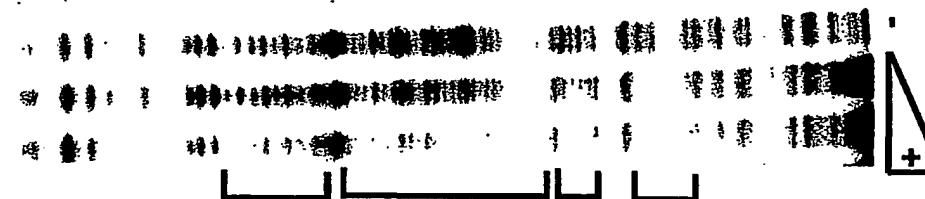
FIG. 5



nt 81- C T A A A T T A A T G T T A T T T T T A A T A A T T A -108



551- AAA 556- AATTAC 582- AAGG 620 640  
CTTGTATTG TAAATTAT  
TCGAT -575 AAAAAATGCT  
-603



633- CAGA 643- ATCACTG 673-678 687-708  
AATA -640 TGTCTAATGA  
ATAATTG -667

A. sar P2

B. sar P1

C. sar P1'

FIG. 6A

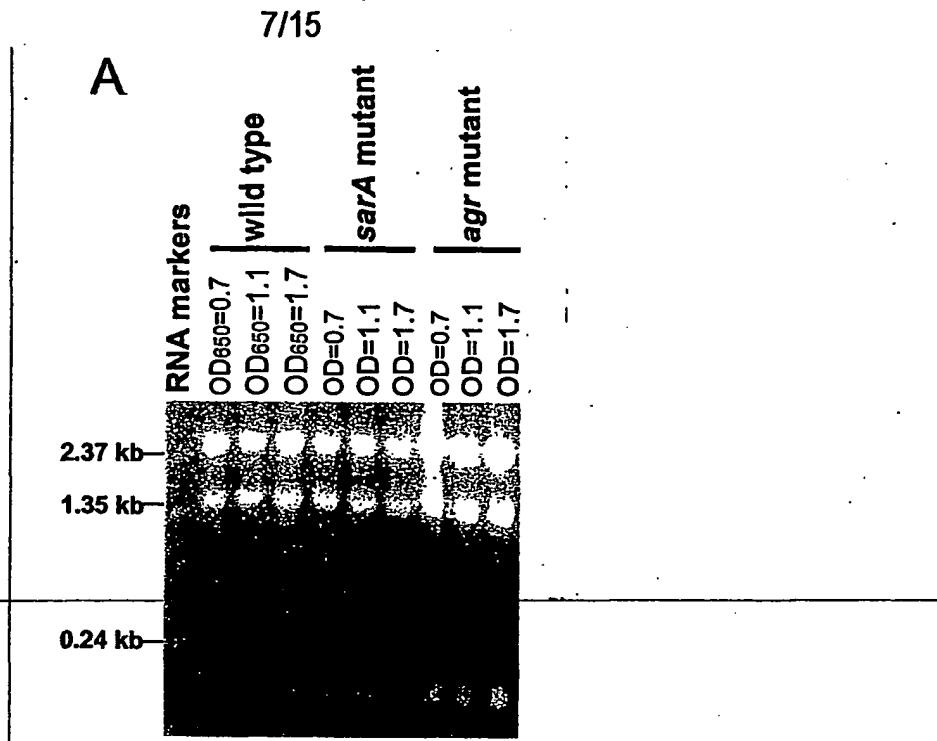
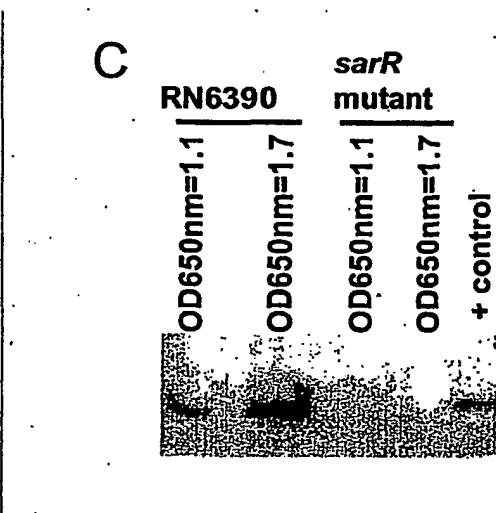


FIG. 6B

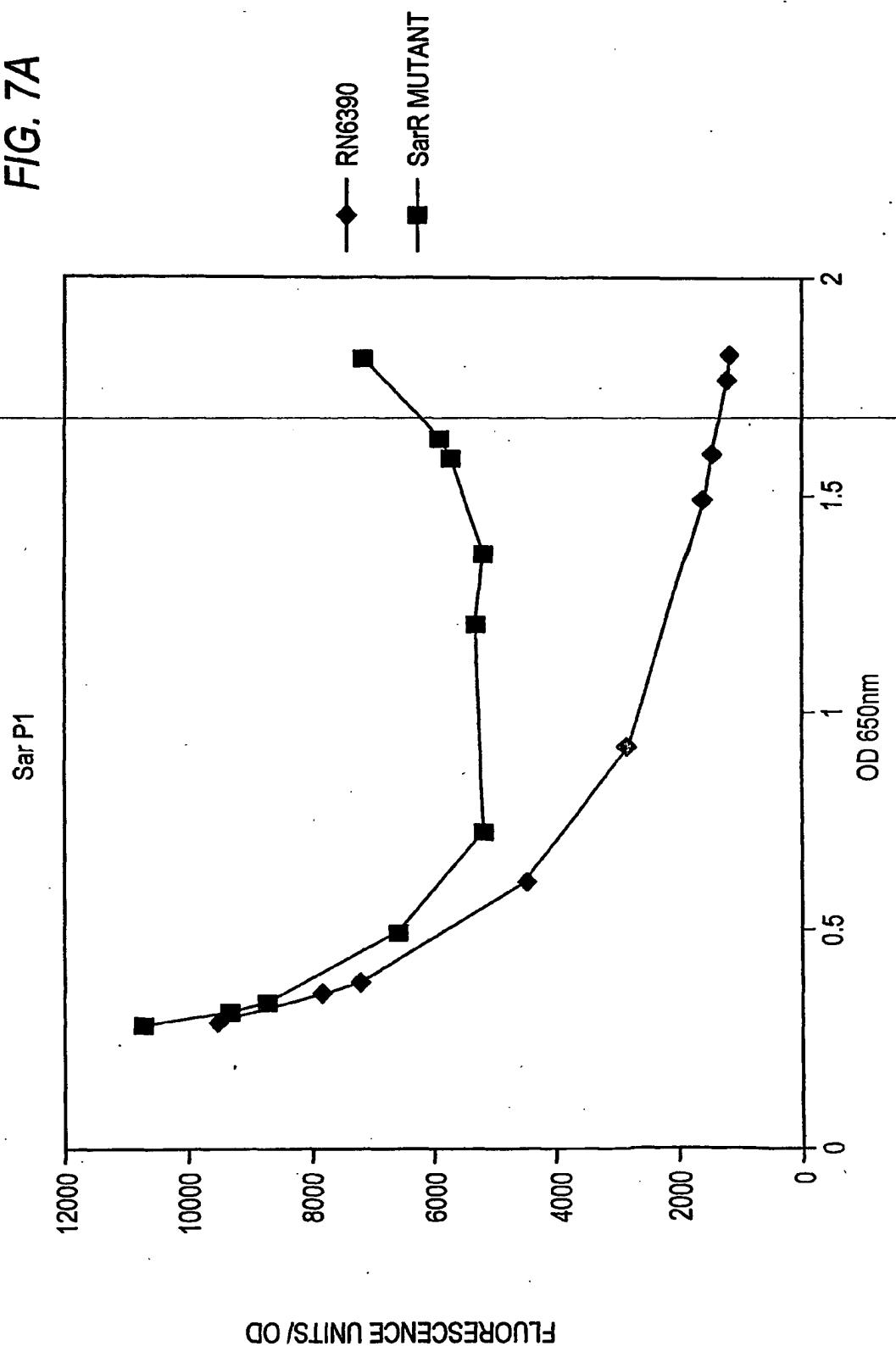


FIG. 6C



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FIG. 7A

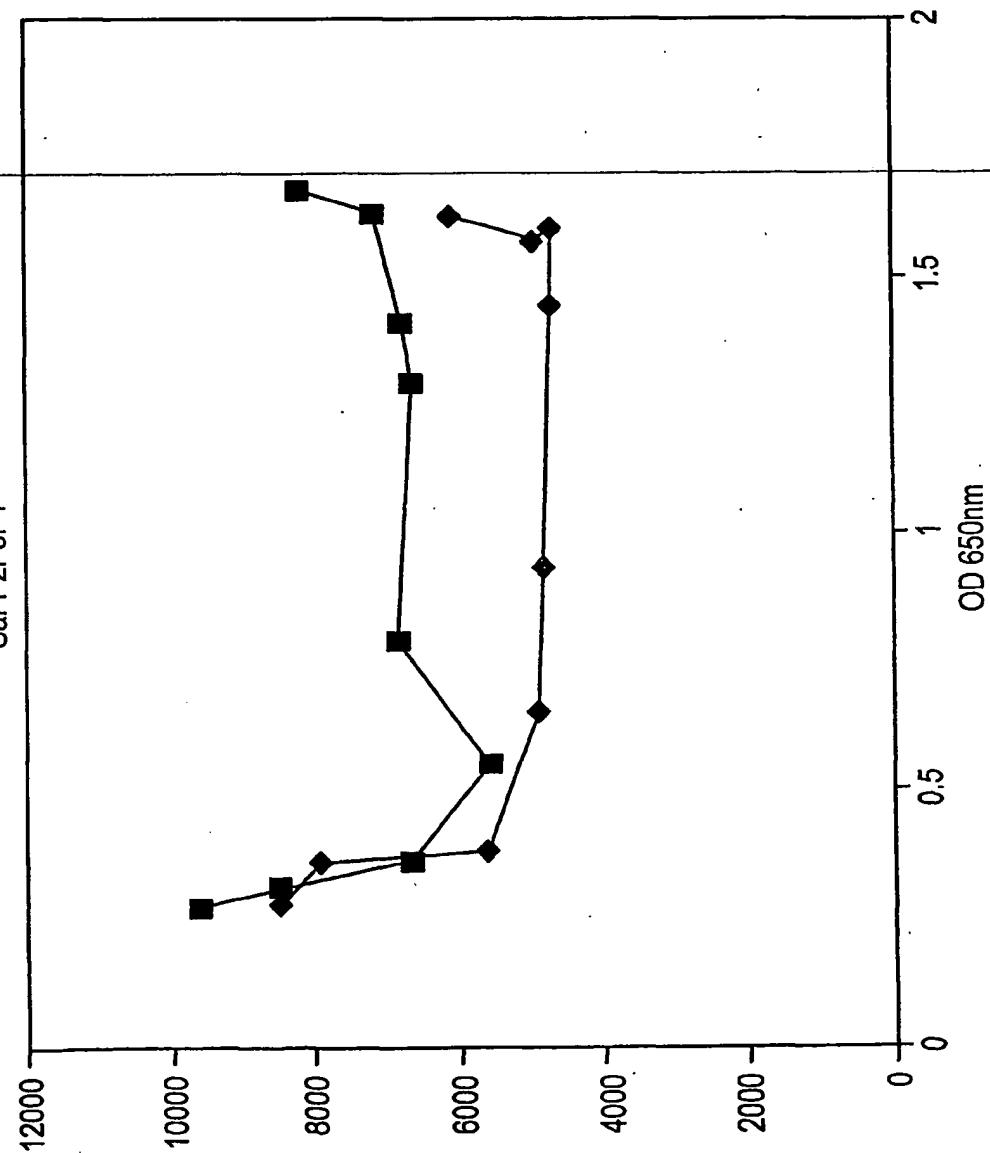


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FIG. 7B

RN6390  
SarR MUTANT



FLUORESCENCE UNITS/OD

SUBSTITUTE SHEET (RULE 26)

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FIG. 8A

**A: *sar* probe**

FIG. 8B

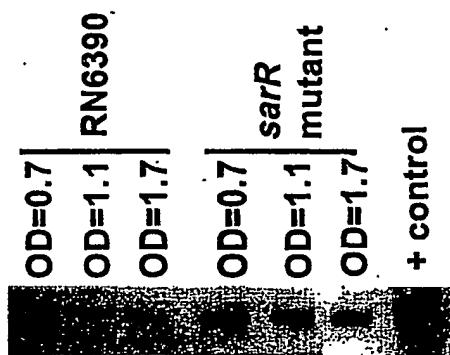
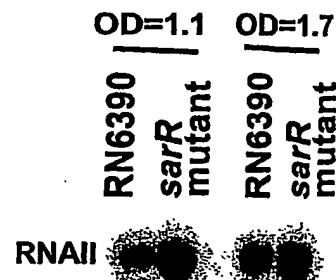
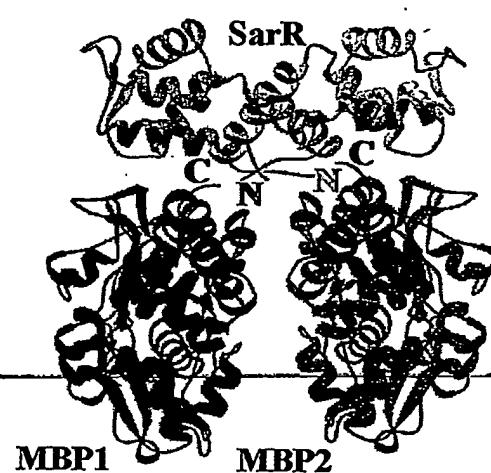
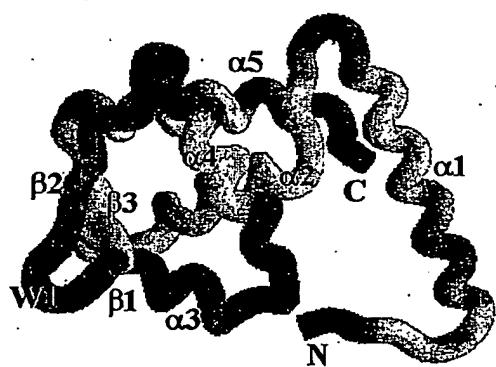
**B: anti-SarA antibody**

FIG. 8C

**C: *agrA* probe**

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**FIG. 9A****FIG. 9B**

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FIG. 10A

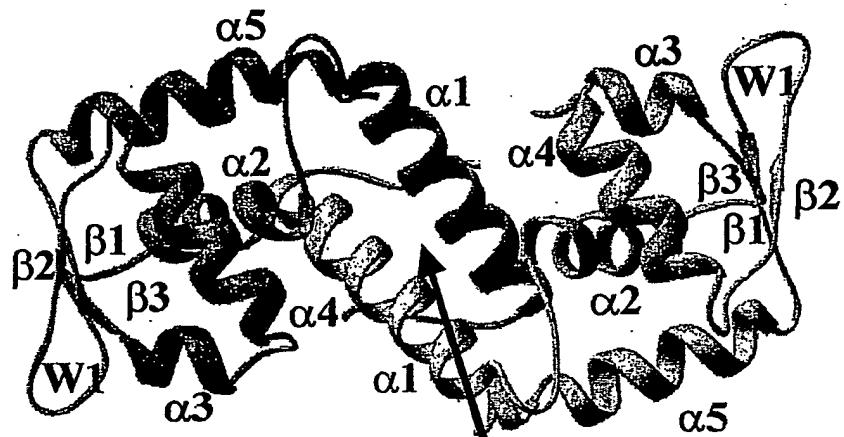


FIG. 10B

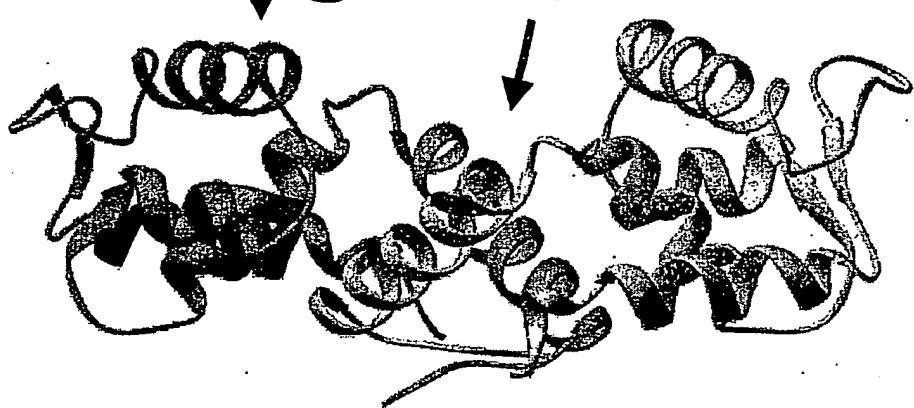
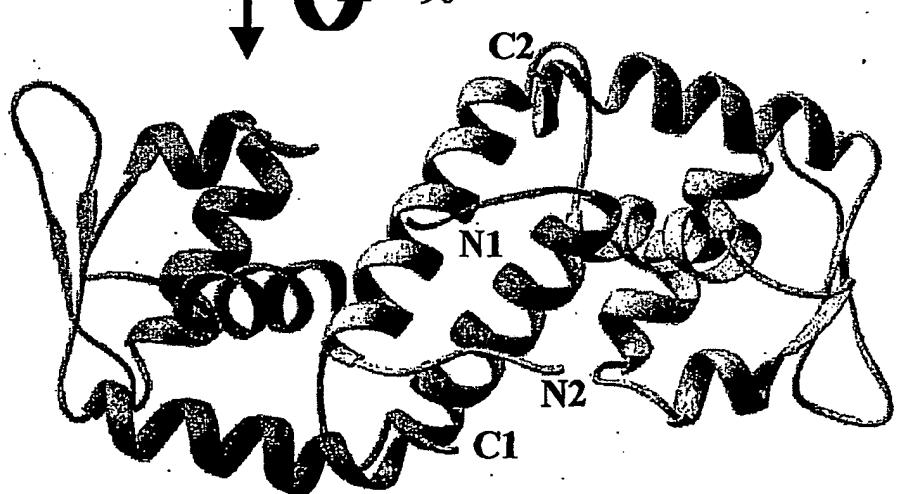


FIG. 10C



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FIG. 11

			<b>α1</b>		<b>α2</b>			
1	M K Y N N H D K I R D F I I I E A Y M F R F K K V K P E V D M T I K E F I L L S F E E F X I L Majority							
1	S Q M I P - K D S K E F L N L M M Y T M Y F K N I I K K H L T L S F V E F T I L SarS1							
1	M A I T K I N D C F E L L S M V T Y A D K I K S L I K K E F S I S F E E F A V L SarA							
1	- M S K I N D I N D L V N A T F Q V K K F F R D T K K K F N I N Y E E I Y I L SarR							
	H AH AHH D		D V T G R I A CAP		H			
			<b>β1</b>	<b>α3</b>	<b>α4</b>			
41	T Y I X X Q X E N E X X L K D I I X X L Y Y K Q P Q L V K A L K X L K K X X Y L Majority							
40	T Y L F H Q Q E N T L P F K K I V S D L C Y K Q S D L V Q H I K V L V K H S Y I SarS1							
41	A I I T S Q N K N I V L L K D L I E T T I H H K Y P Q T V R A L N N L K K Q G Y I SarS2							
39	N H I L R S S E N E I S S K E I A K C S E F K P Y Y L T K A L Q K I L S Q E D Y F SarA							
	D	D	D	D	D	K L K D L K L L SarR		
			<b>β2</b>	<b>β3</b>	<b>β4</b>			
81	S K K R S X D E R T V L I X V X D X Q R X K I E X L L S X V N Q X I K X X N X Majority							
80	S K V R S K I D E R N T Y I S I S E Q R E K T A E R V T L F D Q I I K Q F N - SarS1							
81	I K E R S T E D E R K I L I H M D D A Q Q D H A E Q L L A Q V N Q L L A D - K D SarS2							
79	D K K R N E H D E R T V L I L V N A Q Q R K K I E S L L S R V N K R I T E A N N SarA							
	S K K R S L Q D E R T V I V Y V T D T Q K A N I Q K L I S E L E Y I K N SarR							
	D D D D	D	D	D	D	A H A A H CAP		
	S A - H G - - - - - K T I V V							

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**FIG. 12A & 12B****ELECTROSTATIC POTENTIAL SURFACE OF THE DIMERIC SarR STRUCTURE****BASIC RESIDUE**  
■**ACIDIC RESIDUE**  
■

THE DISTANCE FROM A TO B IS 71 ANGSTROM (20 nt) BUT THE INNER DISTANCE IS 92 ANGSTROM (27 nt)

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FIG. 13A

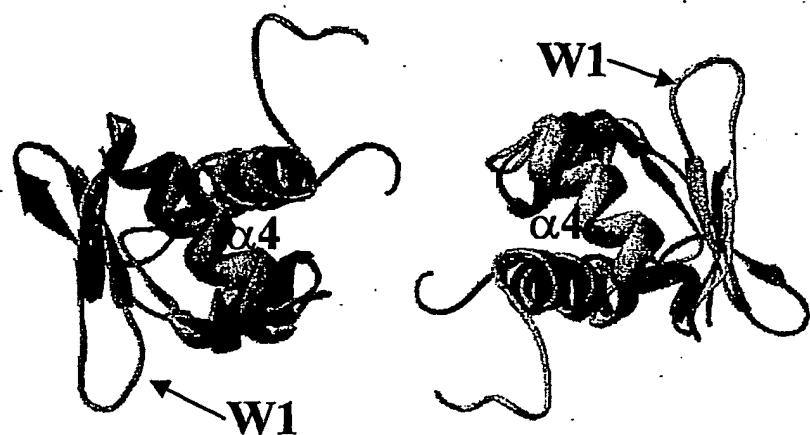


FIG. 13B

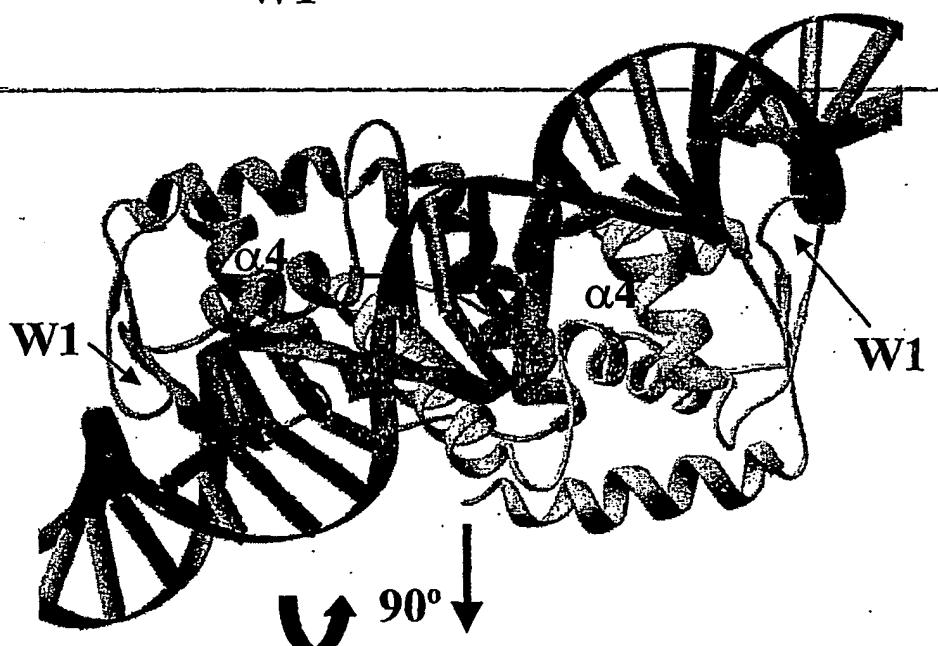


FIG. 13C

